RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF RELATED SUBSTANCES OF ACETAMINOPHEN IN ACETAMINOPHEN, PHENYLEPHRINE HCL AND DEXTROMETHORPHANHBr SOFT GELATIN CAPSULES

Dissertation

Submitted to

THE TAMILNADU Dr. M. G. R. MEDICAL UNIVERSITY,

CHENNAI

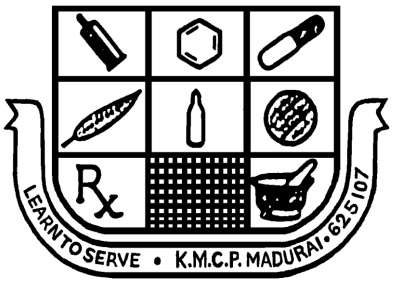
In partial fulfilment for the award of the degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

261230051



DEPARTMENT OF PHARMACEUTICAL ANALYSIS

K. M. COLLEGE OF PHARMACY

MELUR ROAD, UTHANGUDI,

MADURAI – 625107

APRIL-2014

CERTIFICATE

This is to certify that the dissertation entitled **"RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF RELATED SUBSTANCES OF ACETMINOPHEN IN ACETAMINOPHEN, PHENYLEPHRINE HCL AND DEXTROMETHORPHAN HBr SOFT GELATIN CAPSULES"** submitted by **NEELAM VEERAIAH(Reg. No: 261230051**) in partial fulfilment of the degree of Master of Pharmacy in Pharmaceutical Analys is under **The Tamilnadu Dr. M.G.R. Medical University,** Chennai, done at ***K. M. COLLEGE OF PHARMACY,*** MADURAI-625107,is a bonafied work carried out by him under my guidance and supervision during the academic year **2013 - 2014**. This dissertation partially or fully has not been submitted for any other degree or diploma of this university or any other universities.

GUIDE&HOD

Dr. M. Sundarapandian, M. Pharm.,Ph.D.,

Professor and Head**,**

Dept. of Pharmaceutical Analysis,

K. M. College of Pharmacy,

Madurai – 625107.

PRINCIPAL

Dr. S.Venkataraman, M. Pharm.,Ph.D.,

Professor and Head**,**

Dept. of Pharmaceutical Chemistry,

K. M. College of Pharmacy,

Madurai – 625107.

.

CONTENTS

AN EXPRESION OF GRATITUDE

"With God All Things Are Possible"

Milestones in life are achieved, not by individual efforts but by blessings and guidance of elders, near and dear ones of collective wisdom and experience of all those who have shared their views far this project is the product beyond those found within the covers of book. I therefore take this opportunity to express my acknowledgement to all of them.

Let me first thank almighty for giving me life and my parents for educating me and keeping my requirements in priority at all situations. Without their unconditional support and encouragement it would have been impossible to pursue my interest.

It gives me immense pleasure to express my deepest thanks, heartfelt, indebtness and regards to my respected guide, ***Dr. M. SUNDARAPANDIAN., M. Pharm., Ph.D.,*** *Professor and HOD.,* *Dept. of Pharmaceutical Analysis, K. M. College of Pharmacy, Madurai, for providing much of the stimuli in the form of suggestions, guidance and encouragements at all stages of my work.*

I owe my great debt of gratitude and heartfelt thanks to ***Prof. M. Nagarajan., M. Pharm., M.B.A., DMS (IM)., DMS (BM)****., chairman, K. M. College of Pharmacy, Madurai for providing me all the facilities and support for the successful completion of my thesis work.*

I express my deep sincere thanks to ***Dr. S. Venkataraman, M.Pharm., Ph.D.,*** *Principal, professor and H.O.D, Dept. of Pharmaceutical chemistry, for his timely instigation and immeasurable esteemed help to complete this thesis.*

I express my deep sense of gratitude and profound thankfulness to ***Prof. M. S. Prakash., M. Pharm.,*** *Dept. of Pharmaceutical Analysis, K. M. College of Pharmacy, Madurai for his invaluable advice, suggestion and encouragement extended throughout the work.*

I submit sincere thanks and respectful regards to ***Mr. J. NAVANEETHA KRISHNAN, AR&D MANAGER, K. RAGHAVENDRA KUMAR, M. MANI RAJA, and CH. SANTHOSH KUMAR*** *who provided me all the facilities and their excellent guidance for my project and I am also very thankful to staff of industry for their kind cooperation.*

I extend my thanks to ***M. Shanthi.,*** ***B.A, M.Li.Sc.,*** *Librarian and* ***Mrs. M. Sasi kala.,*** ***D.pharm.,*** *Lab Assistant and* ***Mrs. J. Sridevi.,*** *lab attender and all other non teaching staff members of our college for their co-operation.*

I cannot forget to express my gratitude and grateful thanks to my classmates ***Selva ganesh, Ch.Narayana Swamy, Sindhuoor, Sudheer ,Sandhya, Sasi ruba,*** *who helped me directly and indirectly for the successful completion of my project work.*

I owe my thanks to my friends ***B. Jyothi, R. Leena, M. Kathiravn, Sivaraj, Firoz Shaik , Jarugu lakshmi Prasad*** *and my brother for their help during my work.*

.

N. VEERAIAH

ABBREVATIONS

API	-	Active pharmaceutical ingredient
MeOH	-	Methanol
ACN	-	Acetonitrile
RT	-	Retention time
RH	-	Relative Humidity
CI	-	Confidence interval
NMT	-	Not more than
NLT	-	Not less than
NA	-	Not applicable
ND	-	Not detected
Fig	-	Figure
PDA	-	Photo diode array
P^{Ka}	-	Acid dissociation constant
K	-	Partition coefficient
%RSD	-	%Relative standard deviation
RPM	-	Revolutions per minute
ODS	-	Octa decyl silane
K'	-	Capacity factor
N	-	Theoretical plate number/efficiency
LOD	-	Limit of detection
LOQ	-	Limit of quantitation
ICH	-	International Conference on Hormonization
Conc	-	Concentration
Std	-	Standard
ppm	-	Parts per million

ppb	-	Parts per billion
DS	-	Drug substance
DP	-	Drug product
µL	-	Micro liter
µg	-	Micro gram
λ	-	Lambda
µ	-	Micron
mL	-	Milli liter
mg	-	Milli gram
nm	-	Nanometer
Max	-	Maximum
S.no	-	Serial number
^{o}C	-	Degree celsius
pH	-	Hydrogen ion concentration
HPLC	-	High performance liquid chromatography
UV	-	Ultraviolet-Visible

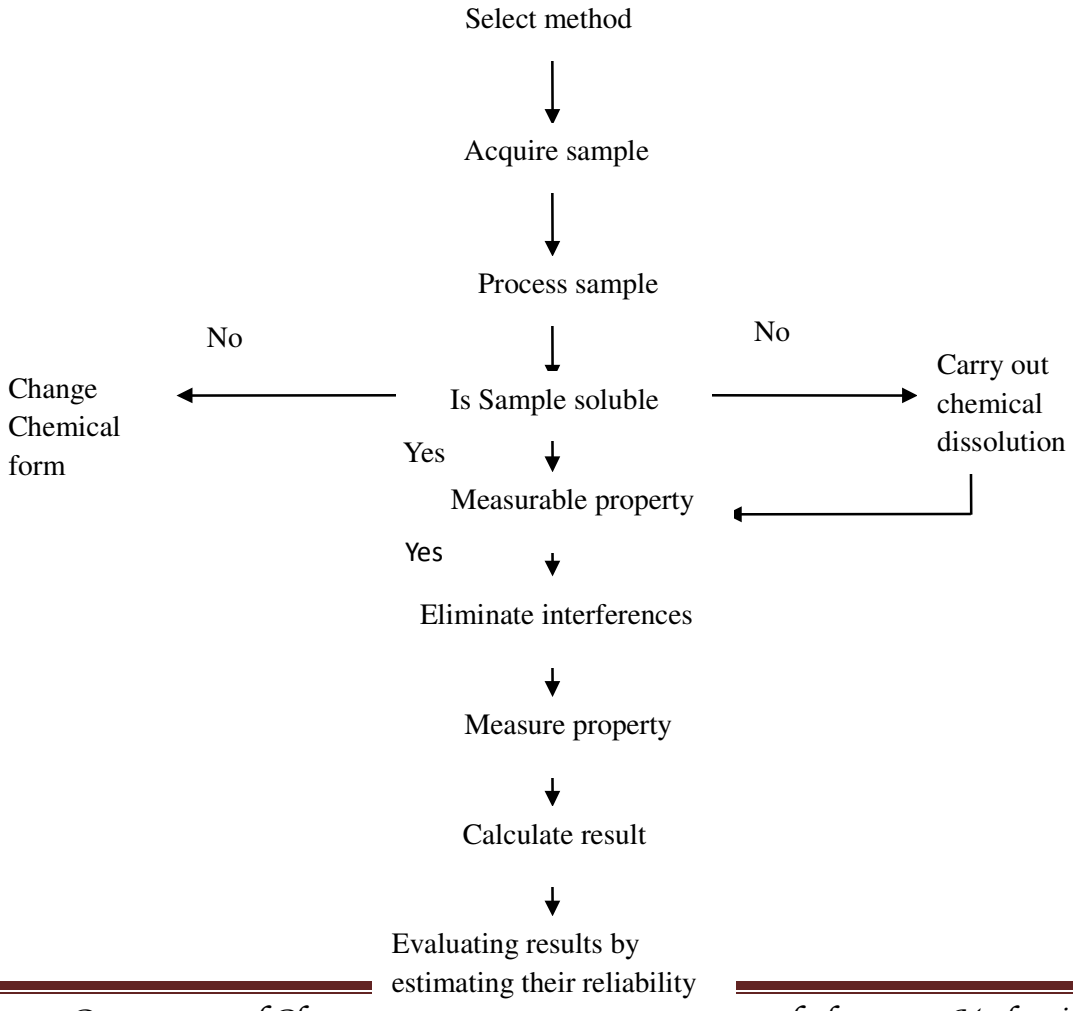
1. INTRODUCTION

Analytical chemistry is measurement science consisting of a set of powerful ideas and methods that are useful in all fields of science and method. it is used to study the chemical composition, structure and behavior of compounds. The purpose of chemical analysis is together and interpret chemical information that will be the value of society in a wide range of contexts. involves the application of a range of techniques and methodologies to obtain and assess qualitative, quantitative and structural information on the nature of components.[1,2]

Qualitative analysis is the identification of elements and compounds present in a sample. Quantitative analysis is the determination of the absolute or relative amounts of elements or compounds present in a sample.

Structural analysis is the determination of the special arrangement of atoms in an element or molecule or the identification of characteristic groups or atoms.

A typical quantitative analysis[2] involves the sequence of steps shown in the following flow diagram (figure-1)



High Performance Liquid Chromatography[3,4,5,6,7]

High performance liquid chromatography is the fastest growing analytical technique for the analysis of the drugs. Its simplicity, high specificity and wide range of sensitivity make it ideal for an analysis of many drugs in both dosage forms and biological fluids. HPLC was developed in the late 1960s and 1970s. Today it is widely accepted separation technique for both sample analysis and purification in variety of areas.

Guide to Liquid Chromatography Mode Selection

Selection of chromatography mode is based upon the analyte polarity, solubility and ionic nature. [7] A guide to liquid chromatography mode selection is represented in figure-2

Figure - 2 Guide to Liquid Chromatography Mode Selection Reverse Phase HPLC

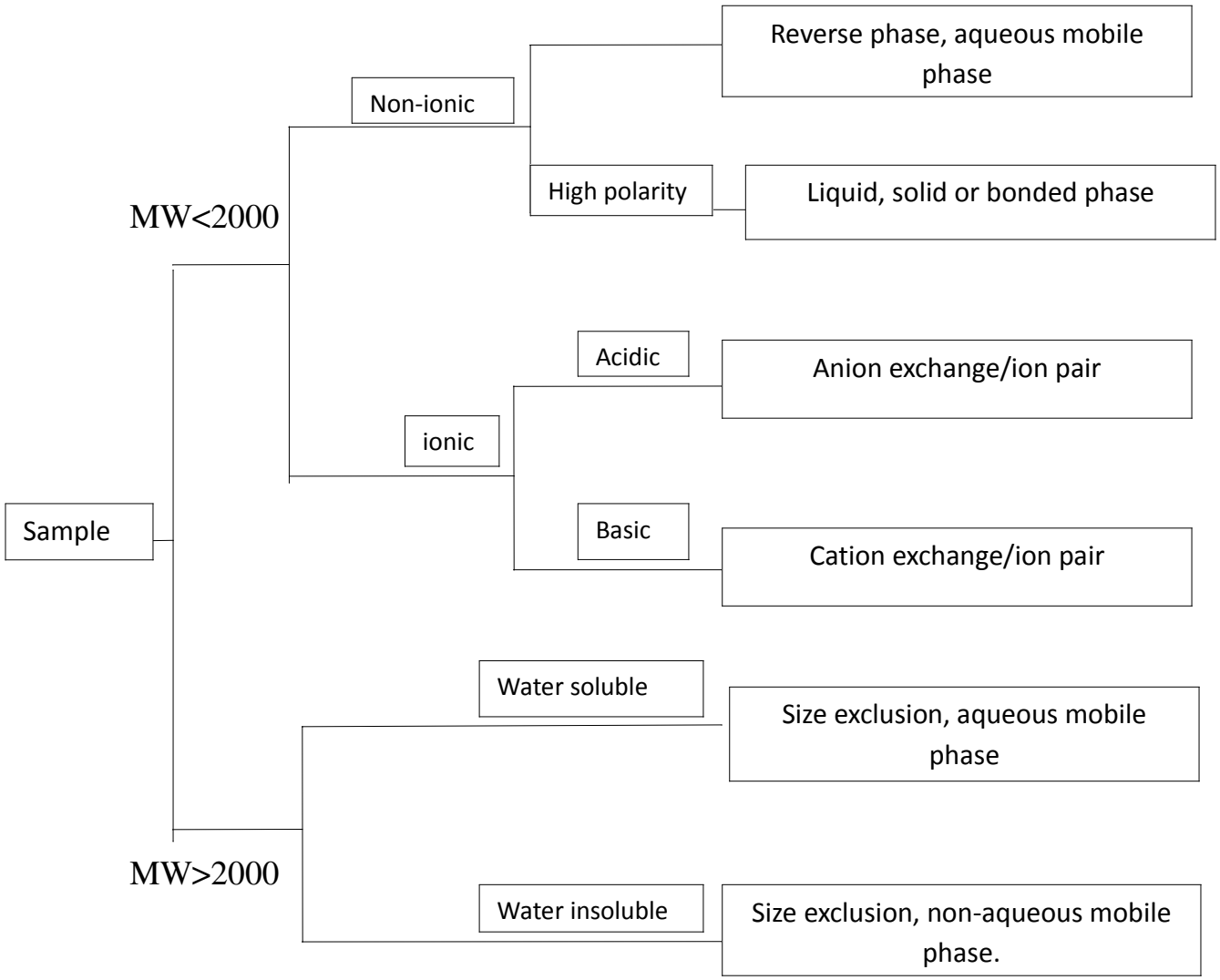
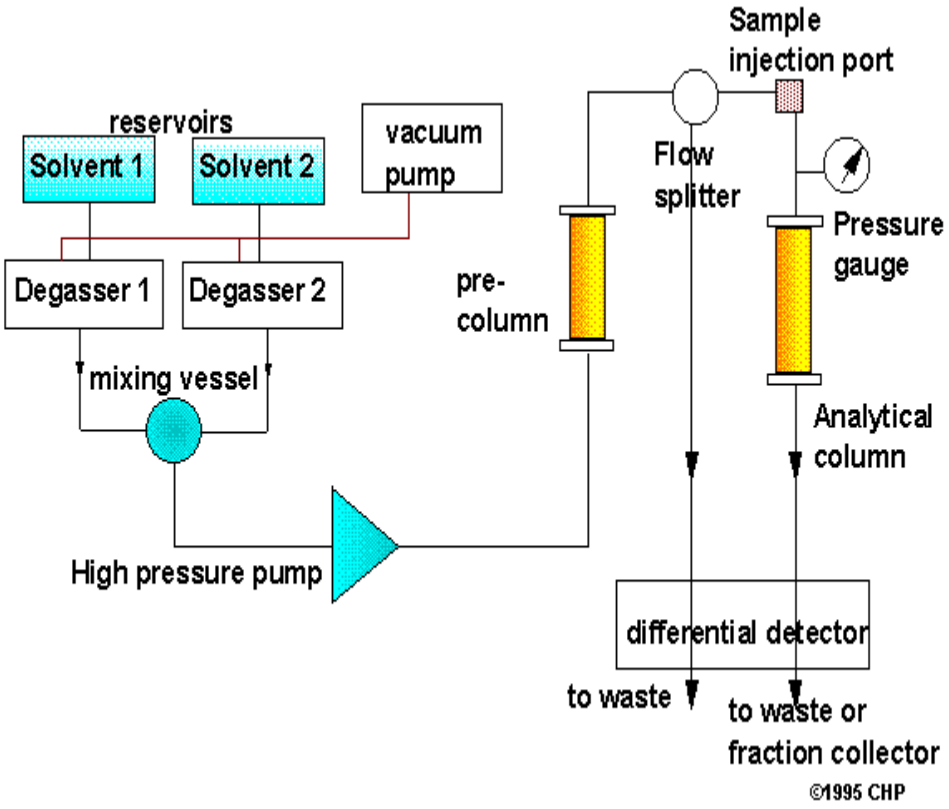


Figure - 3 Block diagram showing the components of an HPLC instrument

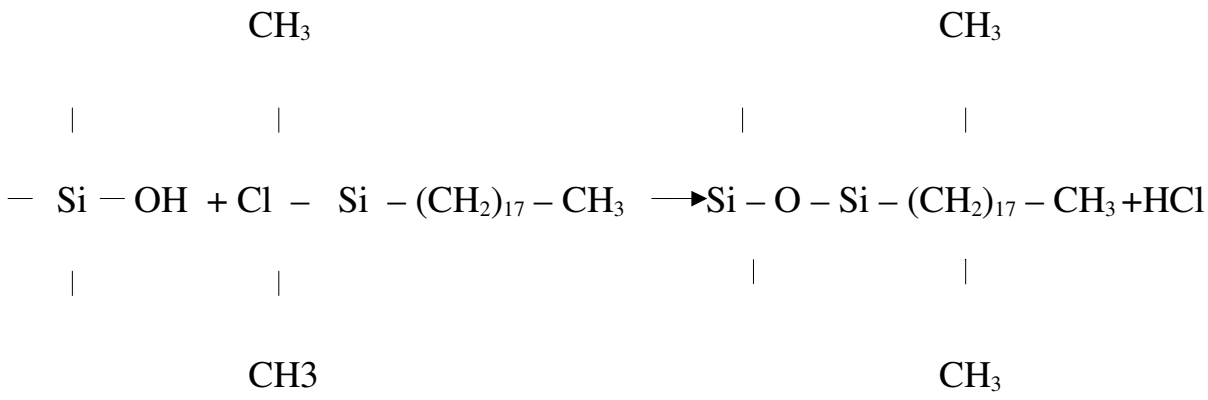


F

Reverse phase column packing

The liquid-liquid partition chromatography uses a stationary phase consisting of liquid layer adsorbed to a surface of silica or alumina. In case of bonded phase or reverse phase HPLC uses a stationary phase consisting of an organic moiety chemically bonded to the surface of silica through the surface silanol groups. Since the organic moieties are generally long-chain hydrocarbons, the mobile phases are generally polar. In this mode, the more polar solutes are eluted first while the non-polar compounds are eluted later. The silanol group can react with a chlorosilane group to form the popular (ODS & C_{18}) octadecylsilane packing.

E.g. Octadecylsilane (ODS or C_{18}).



CH_3

CH_3

For silica based bonded materials, the suitable working pH range is 2 - 8. At pH values of less than 2, the Si-C bond is attached and at the higher pH values, hydrolysis of the siloxane

takes place which leads to degradation or destruction of the packing. In most of the applications of RP-HPLC, elution is carried out with highly polar solvents such as methanol, acetonitrile or tetrahydrofuran in various concentrations. [8]

Reverse phase mobile phase

The primary constituent of RP-HPLC mobile phase is water. Water miscible solvents such as methanol, ethanol, acetonitrile, dioxane, tetrahydrofuran and dimethyl formamide are added to adjust the polarity of the mobile phase. Additionally acids, bases, buffers and/or ionic surfactants are added. The water should be of high quality, either distilled or demineralised water.

The most widely used organic modifiers are methanol, acetonitrile and tetrahydrofuran. Methanol and acetonitrile have comparable polarities but acetonitrile is an aprotic solvent. Ethanol, 1-propanol and 2-propanol are also useful but less polar than methanol. Dioxane, tetrahydrofuran are aprotic solvents that are less polar than acetonitrile. Reverse phase mobile phases are generally non-flammable due to high water content. Degassing is quite important with reverse phase mobile phases.[8]

ANALYTICALMETHOD DEVELOPMENT [1,9]

Establishing an accurate assay procedure for each ingredient of complex dosage formulation containing several therapeutically and chemically compatible drugs with very similar chemical nature is a critical process. The presence of excipients, additives and decomposition products further complicates the analysis. Therefore analytical development is done for few drugs where no compendial method is available.

Method development is done for:

- New drug products
- Already existing products

Methods are developed for new products when no official methods are available and for already existing products to reduce the cost and time for better precision and ruggedness.

STEPS OF METHOD DEVELOPMENT[1]

It starts with the documentation of the developed studies. All the data related to these studies are established and recorded in laboratory notebook.

1. ANALYTICAL STANDARD CHARACTERIZATION

All the known information about the drug or analyte and its structure is collected such as its physical and chemical properties, toxicity, purity, hygroscopic nature solubility and stability.

a. The standard analyte is obtained. Necessary arrangement is made for proper storage in refrigerator, desiccators and freezer.
b. When multiple components are to be analyzed in the sample matrix the number of components are noted, data is assembled and the availability of standards for each one is determined.
c. Special attention to be taken when sample is in less quality.
d. Only the methods which are compatible with sample stability are considered.

2. METHOD REQUIREMENTS

The objectives of method are defined. The required detection limits, linearity, range, accuracy and precision are defined.

3. LITERATURE SEARCH AND RESEARCH METHODOLOGY

Carry out the literature survey for all types of information to the analyte. Literature is done for synthesis, physico-chemical properties, solubility and relevant analytical methods. Books, periodicals, chemical manufacturers and regulatory agency compendia such as USP/NF, AOAC publications are reviewed along with chemical abstract service (CAS) automated computerized literature searches.

a. If any reported methods from the literature are adaptable to the current laboratory setting and future needs are determined.
b. Using information in the literature and prints, methodology is adapted. The methods are modified wherever necessary; acquire additional existing methods for in house analytes and samples.
c. If there are no prior methods for the analytes in the literature, the compounds that are similar and chemical properties are investigated and are worked out.

4. INSTRUMENTAL SET UP AND INITIAL STUDIES

a. The required instrument is set up. Installation, operational and performance of instrumentation using laboratory standard operating procedure are reviewed.
b. Always new consumables (solvents, filter and gases) are used.

c. The analyte standard in a suitable injection/introduction solution and in known concentration and solvents are prepared. It is important to start with an authentic, known standard rather than with a complex sample matrix. If the sample is extremely close to the standard (example: bulk drug) then it is possible to start work with the actual sample.
d. Feasibility of method with regards to the analytical figures of merit obtained is evaluated.

5. OPTIMIZATION

During optimization one parameter is changed at a time and set of conditions are isolated rather than using a trial and error approach. Work has been done from an organized methodological plan and every step is documented in case of dead ends.

6. DOCUMENTATION OF METHOD DEVELOPMENT WITH ACTUAL SAMPLE

The sample solution should lead to absolute identification of the peak of interest apart from all other matrix components.

7. EVALUATION OF METHOD DEVELOPMENT WITH ACTUAL SAMPLE

The sample solution should lead to absolute identification of the peak of interest apart from all other matrix components.

8. DETERMINATION OF PERCENT RECOVERY OF ACTUAL SAMPLE AND DEMONSTRATION OF QUANTITATIVE SAMPLE ANALYSIS.

Percentage recovery was determined by adding authentic standard analyte into a sample matrix. Reproducibility of recovery from sample to sample and has been determined. It is not necessary to obtain 100% recovery as long as the results are reproducible and known with high degree of certainty.

ANALYTICAL METHOD VALIDATION[10, 11, 12, 13, 35, 36]

Validation is a key process for effective Quality Assurance "Validation is establishing documented evidence which provides a high degree of assurance that a specific process or equipment will consistently produce a product or result meeting its predetermined specifications and quality attributes.

The validation guidelines recommended from ICH (International Conference on Harmonization) consists characteristics for consideration during the validation of analytical procedures included as part of registration applications within EC, Japan and USA.

Type of analytical procedures to be validated

Validation of analytical procedures is directed to the four most common types of analytical procedures.

1. Identification test.
2. Quantitative test for impurities content.
3. Limit test for the control of impurities
4. Quantitative test of the active moiety in samples of drug substance on drug product on other selected components in the drug product.

Assay procedures are intended to measure the analyte present in given sample, assay represent a quantitative measurement of the major component(s) in the drug sample.

Objective of validation[14, 15]

The primary objective of validation is to form a basis for written procedure for production and process control which are designed to assure that the drug products have the identity, strength, quality, purity, safety and efficacy. Each step of the manufacturing process must be controlled to maximize the probability that the finished products meet all quality and design specification.

Benefits of Validation:

a. Produces quality products.
b. Helps in process improvement technology transfer, related product validation, failure investigation, and increased employee awareness.
c. Cost reduction by increasing efficacy, few reject, longer equipment life, production of cost effective products.
d. Helps in optimization of process or method.
e. Regulatory affairs department approved the products for export.

VALIDATION AS DEFINED BY DIFFERENT AGENCIES

1. USFDA:

According to this "Validation is the process of establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality attributes.

2. WHO:

Defines Validation as an action of providing any procedure, process, equipment, material, activity or system actually leads to the expected results.

3. EUROPEAN COMMITTEE:

Defines Validation as an action of providing in accordance with the principles of GMP that any procedure, process, material, activity or system actually lead to expected results. This process consists of establishment of the performance characteristics and the limitations of the method.

Method performance parameters are determined using equipment that is:

1. Within specification.
2. Working correctly.
3. Adequately calibrated.

Method validation is required when:

1. A new method is developed.
2. Revision of established method.
3. When established methods are used in different laboratories and by different analysts.
4. Comparison of methods.
5. When quality control indicates method changes.

Typical analytical parameters used in assay validation include:

- Precision
- Accuracy
- Linearity
- Range
- Ruggedness
- Robustness
- Limit of detection
- Limit of quantitation
- Selectivity
- Specificity

PRECISION

A) Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurement obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision of an analytical procedure is usually expressed at the variance, standard deviation or coefficient of variation of a series of measurements.

Validation of tests for assay and for quantitative determination of impurities includes an investigation of precision.

Precisionisdeterminedbyusingthemethodtoassayasampleforasufficientnumberof times to obtain statistically valid results (i.ebetween6-10).

Theprecisionisthen expressedastherelativestandarddeviation

$$\%RSD = \frac{STDdevx100\%}{Mean}$$

Repeatability

It express the precision under the same operating conditions over a short interval of time. Repeatability is also termed as intra - assay precision. It should be assessed using a minimum of nine determinations covering the specified range for the procedure (e.g. three concentration/three replicates each) or a minimum of determinations at 100% of the test concentration.

Intermediate precision

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analysts, equipment, etc.

Reproducibility

Reproducibility is assessed by means of an inter-laboratory trial. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance inclusion of procedures in Pharmacopoeias.

ACCURACY

"Accuracy is a measure of the closeness of test results obtained by a method to the true value."

Accuracy indicates the deviation between the mean value found and the true value. It is determined by applying the method to samples to which known amounts of analyte have been added. These should be analyzed against standard and blank solutions to ensure that no interference exists. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay. Accuracy and precision are not the same, as the diagram below indicates. A method can have good precision and yet not be accurate.

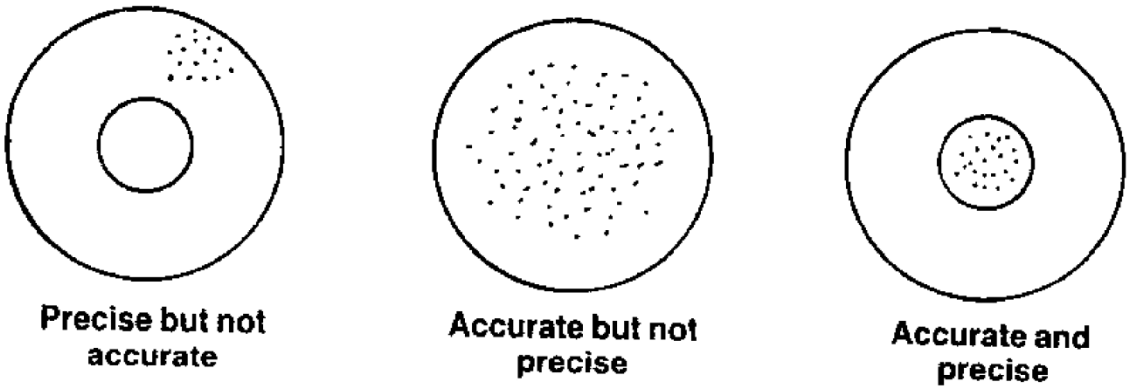


Figure-4

Errors in measurement can be divided into two general categories: systematic errors and random errors.

Systematic errors result from sources that can be traced to the methodology, the instrument or the operator and affect both the accuracy and the precision of the measurement.

Random errors only affect the precision and are difficult to eliminate, because they are the result of random fluctuations in the measured signal, due to noise and other factors.

Whilst systematic errors are proportional to the sum of individual contributions, random errors are proportional to the root of the sum of the squares of the individual contributions. Thus, the imprecision of the entire procedure is often dominated by the random errors of the most imprecise step.

LINEARITY

This is the method's ability to obtain results which are either directly, or after mathematical transformation proportional to the concentration of the analyte within a given range. Linearity is determined by calculating the regression line using a mathematical treatment of the results (i.e. least mean squares) vs. analyte concentration.

RANGE

The range of the method is the interval between the upper and lower levels of an analyte that have been determined with acceptable precision, accuracy and linearity. It is determined on either a linear or nonlinear response curve (i.e. where more than one range is involved, as shown below) and is normally expressed in the same units as the test results.

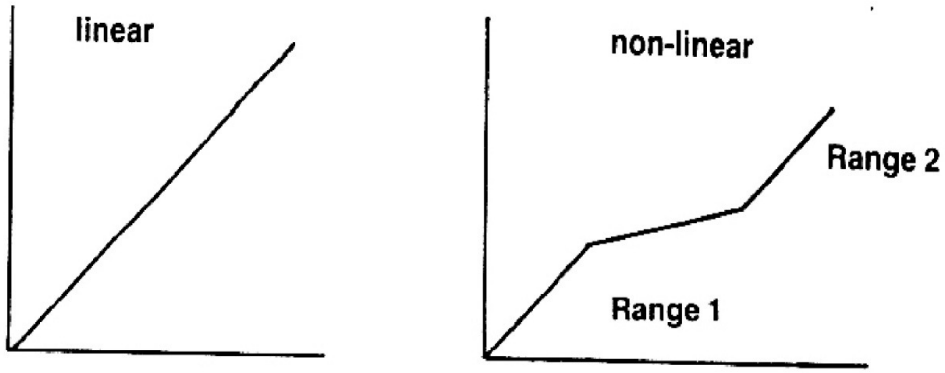


Figure- 5

RUGGEDNESS

Ruggedness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions i.e. different analysts, laboratories, instruments, reagents, assay temperatures, small variations in mobile phase, different days etc. (i.e. from laboratory to laboratory, from analyst to analyst.)

ROBUSTNESS

Robustness is depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. It is the measure of capacity of an assay to remain by small but deliberate variations in method parameters and provide an indication of its reliability in normal usage degradation and variation in chromatography column, mobile phases and inadequate method development are common causes of lack of robustness.

Examples of typical variations are

- Stability of analytical solutions
- Extraction time
- Influence of variation of pH in a mobile phase
- Influence of variation in mobile phase composition
- Different columns
- Temperatures
- Flow rate

LIMIT OF DETECTION

This is the lowest concentration in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. The limit of detection is important for impurity tests and the assays of dosages containing low drug levels and placebos.

The limit of detection is generally quoted as the concentration yielding a signal-to-noise ratio of 2:1 and is confirmed by analyzing a number of samples near this value (6) using the following equation. The signal-to-noise ratio (5) is determined by:

$s = H/h$

Where H = height of the peak corresponding to the component

h = absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution.

Since the limit of detection is dependent on the signal-to-noise ratio, it can be improved by enhancing the analyte signal and reducing the detector noise. The signal (i.e. peak height) can be increased by selecting the optimum monitoring wavelength, increasing the injection volume or mass (below signal or column saturation), increasing the peak sharpness with high efficiency columns and by optimizing the mobile phase. For absorbance detectors, longer path lengths in the flow cell enhance sensitivity though often to the detriment of post column dispersion.

Noise can be reduced by using high sensitivity detectors with low noise and drift characteristics, slower detector response time, mobile phases with low absorbance and pumps with low pulsation.

LIMIT OF QUANTITATION

This is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy.

It is quoted as the concentration yielding a signal-to-noise ratio of 1 0: 1 and is confirmed by analyzing a number of samples near this value.

SELECTIVITY AND SPECIFICITY

Selectivity is the ability to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the sample matrix.

Specificity for an assay ensures that the signal measured comes from the substance of interest and that there is no interference from excipients and/or degradation products and/or impurities.

Determination of this can be carried out by assessing the peak identity and purity.

Diode array detectors can facilitate the development and validation of HPLC assays. Spectral data obtained from diode array detectors, effectively supplement the retention time data for peak identification, also spectral manipulation often provides information about the peak purity. The table below lists several of the techniques available for assessing peak identity and purity.

The purity index is a measure of the peak's relative purity, measured using a full comparison of spectral data for the leading and training edge of the peak Figure 4. A value of 1.5 is commonly accepted to indicate a pure peak but >1.5 would indicate the presence of an impurity, (9) as shown in Figure 6 & 7.

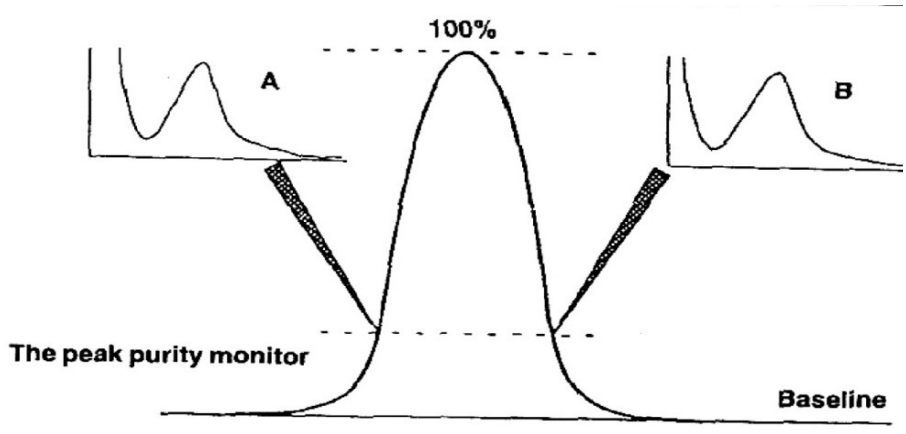
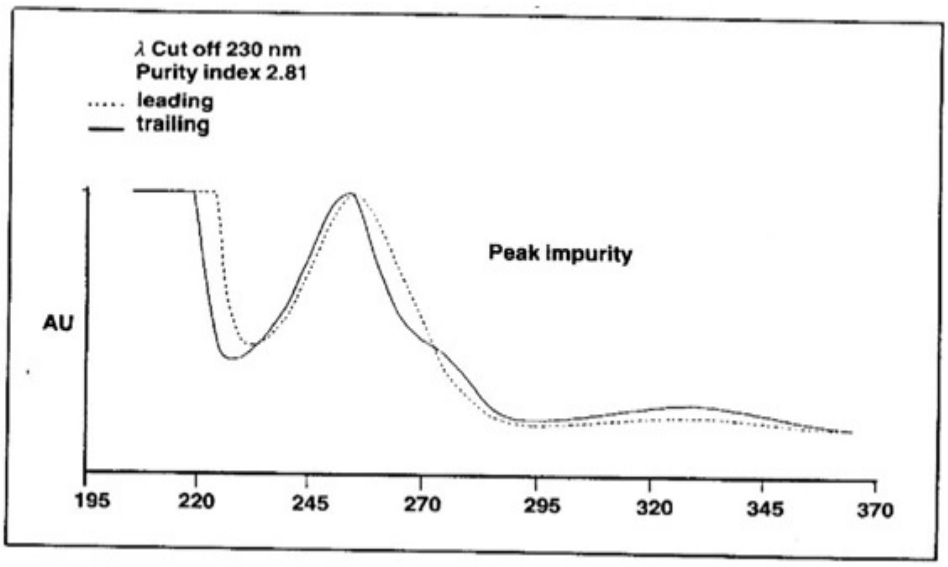


Figure- 6

Spectral comparison showing peak purity



Comparison of reference versus sample apex spectra for peak identity

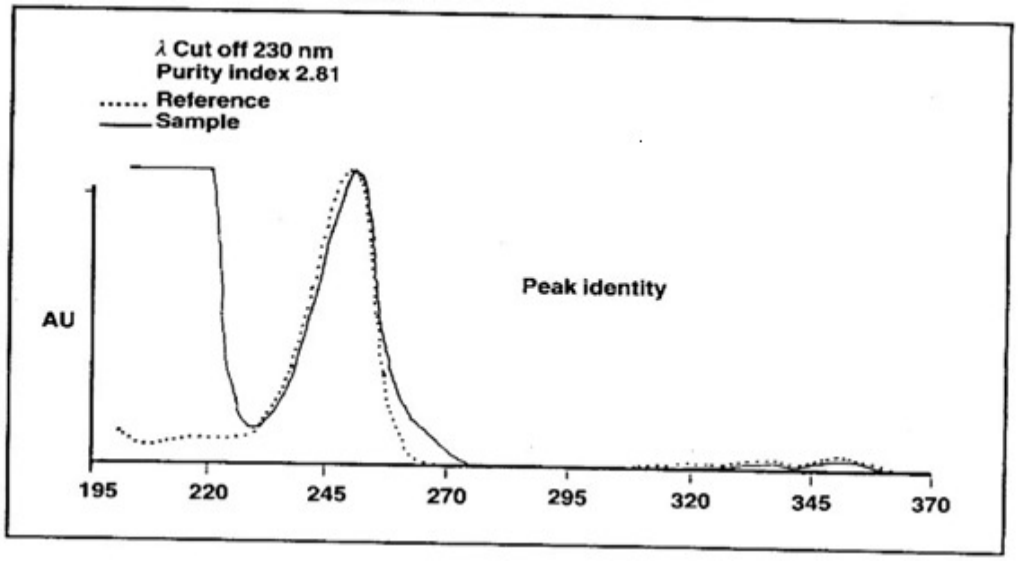


Figure-7

SYSTEM SUITABILITY TESTS (SST)

Once a method or system has been validated the task becomes one of routinely checking the suitability of the system to perform within the validated limits.

The simplest form of an HPLC system suitability test involves a comparison of the chromatogram trace with a standard trace. This allows a comparison of the peak shape, peak width, and baseline resolution.

Alternatively these parameters can be calculated experimentally to provide a quantitative system suitability test report:

1. Number of theoretical plates (efficiency)
2. Capacity factor
3. Separation (relative retention)
4. Resolution
5. Tailing factor
6. Relative Standard Deviation (Precision)

These are measured on a peak or peaks of known retention time and peak width.

1. Plate number or number of theoretical plates (n)

This measures the sharpness of the peaks and therefore the efficiency of the column. This can be calculated in various ways, for example the USP uses the peak width at the base and the BP at half the Height.

An equation used to calculate 'N' is

$$\mathbf{N = 5.54[t/w_{h/2}]^2}$$

Where

$W_{h/2}$ = peak width at 1/2 peak height Wb = peak width at base

t = retention time of peak

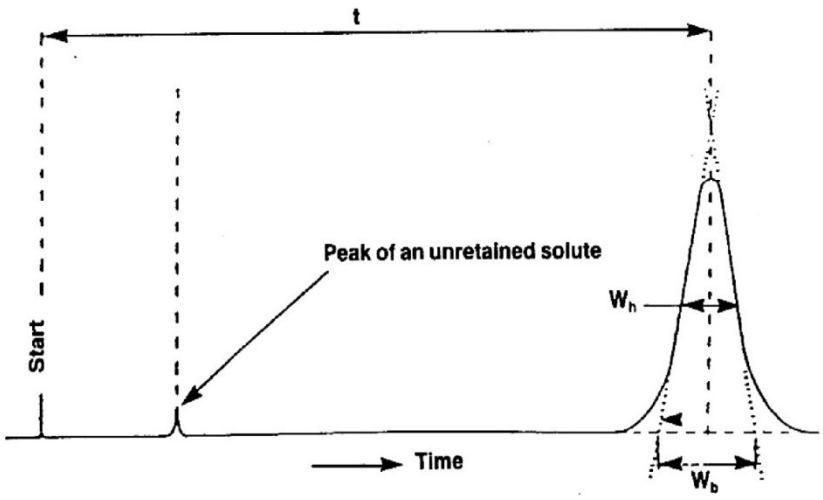


Figure- 8

Therefore the higher the plate number the more efficient the column.

The plate number depends on column length - i.e. the longer the column the larger the plate number. Therefore the column's efficiency can also be quoted as:

Either- as the plate height (h), or the height equivalent to one theoretical plate (HETP).

h= L/n

Where L = length of column n

Or - as plates/meter.

2. Capacity factor (capacity ratio) k

This value gives an indication of how long each component is retained on the column (i.e. how many times longer the component is retarded by the stationary phase than it spends in the mobile phase).

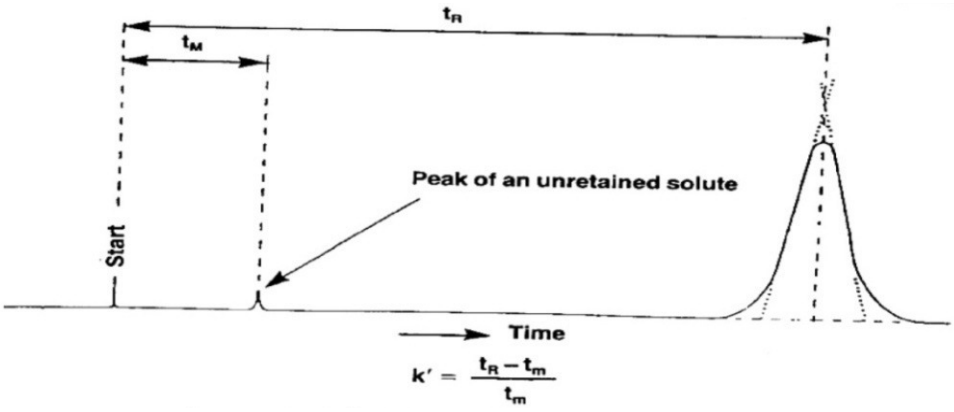


Figure- 9

k' is used in preference to retention time because it is less sensitive to fluctuations in chromatographic conditions (i.e. flow rate) and therefore ensures greater reproducibility from run to run. In practice the k value for the first peak of interest should be >l to assure that it is separated from the solvent.

3. Separation Factor (relative retention)

This describes the relative position of two adjacent peaks. Ideally, it is calculated using the capacity factor because the peaks' separation depends on the components' interaction with the stationary phase.

Therefore considering peaks A and B

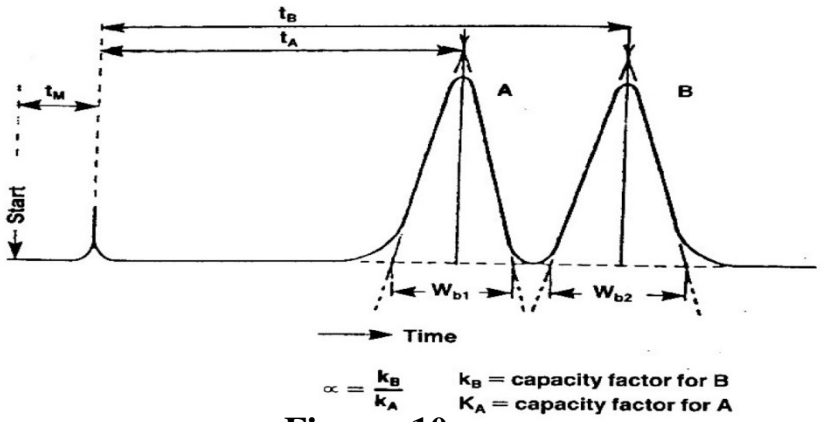


Figure- 10

k for the later peak is always placed in the numerator to assure a value >l.

If the capacity factor is used then the separation factor should be consistent for a given column, mobile phase, composition and specified temperature, regardless of the instrument used.

4. Peak Resolution R

This is not only a measure of the separation between two peaks, but also the efficiency of the column. It is expressed as the ratio of the distance between the two peak maxima. (At) to the mean value of the peak width at base (Wb).

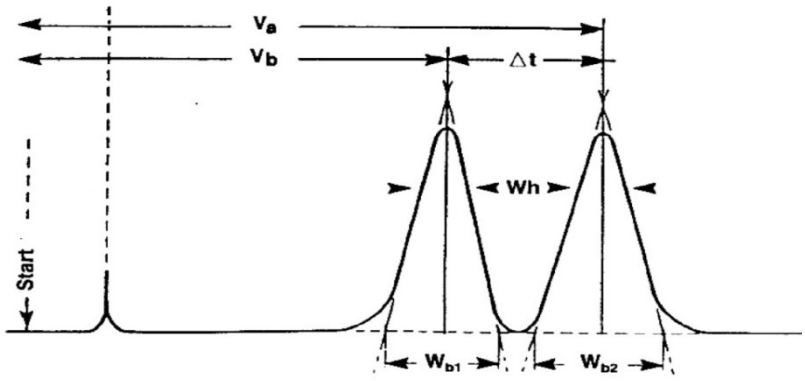


Figure- 11

5. Tailing Factor T

This is a measure for the asymmetry of the peak.

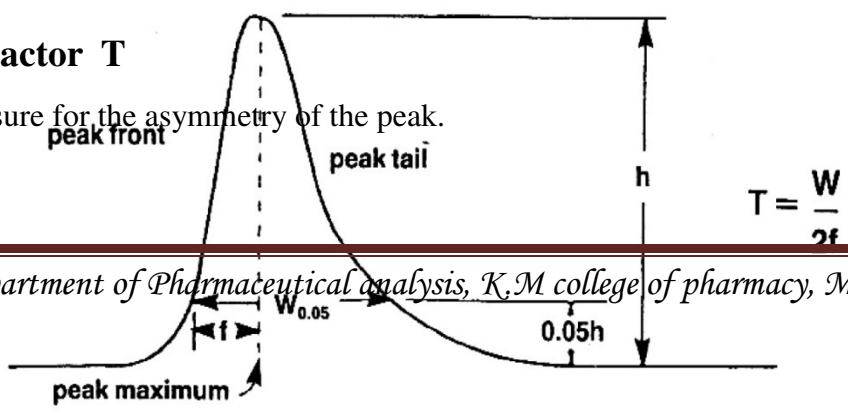


Figure- 12

6. Relative Standard Deviation or precision

For an HPLC system this would involve the reproducibility of a number of replicate injections (i.e. 6) of an analytical solution.

The USP requires that unless otherwise specified by a method:

if a relative standard deviation of <2% is required then five replicate injections should be used. if a relative standard deviation of >2% is required then six replicate injections should be used Factors which could affect the precision of an HPLC system are shown in table 1.

Table No. 1

Precision	**Controlling Factors**
Retention time	Pump flow and composition precision Column temperature Mobile phase composition
Peak area	Auto sampler: inj mode, inj volume Pump: flow, pulsation Detector: noise and drift, response Data system: sampling rate, integration parameters

In most cases the system's Relative Standard Deviation is required; deciding which of the

other tests are required is not straightforward. To assist with the decision it has been suggested that those parameters which have an effect on the system precision should be used. For instance the resolution of two peaks with similar retention times should be quoted, because, if it is below a critical value, the precision will be affected.

In addition "diode array" detectors allow for the determination of the relative purity factor typically called: Peak Purity.

The retention time precision is important, because not only is retention time the primary method for peak identification, but also variations can indicate problems within the LC system (i.e. with the piston seals, check valves etc). Use of a column oven can overcome laboratory temperature variations, which is the most common cause of retention time drift.

The most dominant factor controlling the repeatability of peak area is the auto sampler's precision, though the effect of noise and integration parameters will become more significant with small peaks

METHODS USED FOR THE EXAMINATION OF PHARMACEUTICAL MATERIAL MAY BE BROADLY CLASSIFIED AS:

CLASS A: Tests designed to establish identity, whether of bulk drug substances or of a particular ingredient in a finished dosage form.

CLASS B: Methods designed to detect and quantitate impurities in a bulk drug substance or finished dosage form.

CLASS C: Methods used to determine quantitatively the concentration of a bulk drug substance or of a major ingredient in a finished dosage form,

CLASS D: Methods used to assess the characteristics of finished dosage forms such as dissolution profile and content uniformity.

2. PROFILE

2.1 DRUG PROFILE

i. ACETAMINOPHEN[16]

Category **:** Analgesic and antipyretic**.**

Nomenclature

IUPAC NAME **:** *N*-(4-Hydroxyphenyl) acetamide.

synonyms **:** Paracetamol

Chemical Structure of Acetaminophen

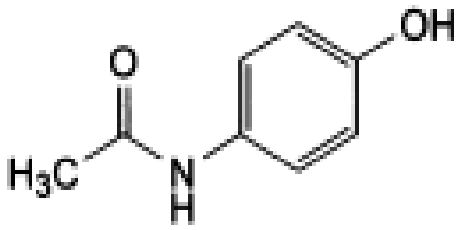


Fig-12 Structure of **Acetaminophen**

Molecular Formula **:** $C_8H_9NO_2$

Molecular mass **:** 151.2 g/mol

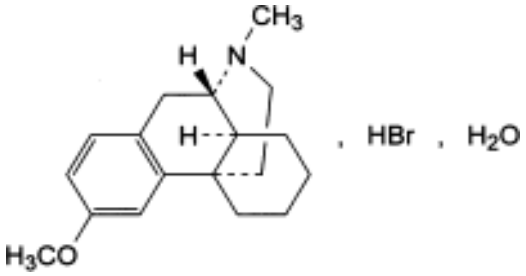
Solubility **:** Sparingly soluble in water, freely soluble in alcohol, very slightly soluble in ethylene chloride.

Melting point **:** 168 ℃ to 172 ℃

ii. DEXTROMETHORPHAN HYDRO BROMIDE[17]

IUPAC name: Ent -3-Methoxy-17-methylmorphinan Hydrobromide monohydrate.

Chemical Structure of Dextromethorphan Hydro Bromide

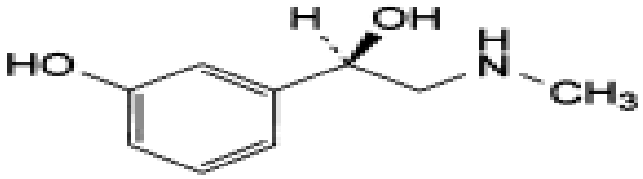


Molecular Formula	**:**	$C_{18}H_{26}BrNO, H_2O$
Molecular Weight	**:**	370.3 g / mol
Appearance	**:**	Almost white, crystalline powder.
Solubility	**:**	Sparingly soluble in water, freely soluble in alcohol.
Melting point	**:**	About 125 °C, with decomposition.
Action and use	**:**	Cough suppressant.

iii. **PHENYLEPHRINE HCL**[16]

Molecular Formula **:** $C_9H_{13}NO_2.HCL$

Chemical Structure of Phenylephrine HCl



.HCL

Molecular Weight **:** 203.67 g / mol

IUPAC name **:** 1*R*)-1-(3-hydroxyphenyl)-2-(methylamino) ethanol. Hydrochloride

Appearance **:** A white or almost white, crystalline powder

Solubility **:** Slightly soluble in water, sparingly soluble in methanol, slightly soluble in alcohol. It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides.

Melting point **:** 174 °C.

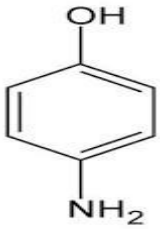
Action and use **:** Sympathomimetic.

2.2IMPURITIES DRUG PROFILE

i. 4 – AMINOPHENOL[18]

Molecular Formula **:** C_6H_7NO

Chemical Structure of 4-Aminophenol

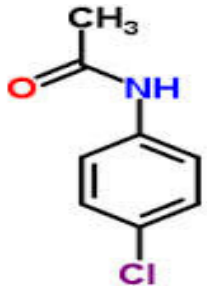


Molecular Weight	**:**	109.13 g / mol
IUPAC name	**:**	4-Amino 1- Hydroxy Benzen.
Appearance	**:**	Crystalline powder, gradually become darker.
Solubility	**:**	Very soluble in water, soluble in alcohols
Melting point	**:**	186 °C.
Action and use	**:**	Photographic developer .

ii. 4 – CHLOROACETANILIDE[18]

Molecular Formula : C_8H_8ClNO.

Chemical Structure of 4-chloroacetanilide



Molecular Weight : 169.60 g / mol

IUPAC name : N-(4-chloro phenyl) acetamide.

Appearance : Orthorombic crystals.

Solubility : Practicallysoluble in water, freely soluble in alcohols, ether, carbon disulphide, slightly soluble in carbon tetra chloride.

Melting point : 178 – 179°C.

3. LITERATURE REVIEW

D. B. Shepherd et al.,[19] have developed stability indicating HPLC method for the determination of acetaminophen and its related substance.

Column	: Restek Ultra II® Pentafluorophenyl column (150mm X 4.6mm, 5um)
Mobile phase A (90:5:5)	: 10mM ammonium acetate buffer/methanol/acetonitrile
Mobile phase B	: Methanol and Acetonitrile (50:50)
Wavelength detection	: UV detector at 272nm
Gradient program	: 100% Mobile Phase A for 7 minutes 60% Mobile Phase B at 12minutes

The method was linear, accurate and precised for 4-aminophenol and other unknown impurities between 0.25ug/mL to 5ug/mL(0.05% - 1.0% Acetaminophen).

Badea1*et al.,[20] were developed and validated Reverse Phase High Performance liquid Chromatography (RP-HPLC) method for the estimation of acetaminophen and it's main impurities 4-nitrophenol, 4-chloroacetanilide as well as 4-aminophenol and it's degradation products p-benzoquinone and hydroquinone.

Column	: Hypersil Duet C_{18}/SCX column
Mobile phase	: Phosphate buffer (pH 5& 4.88) and methanol
Gradient program	: Using gradient elution

The limits of detection and quantification was established to be lower than 0.1 mg/mL and 0.5 mg/mL, respectively. The method was successfully applied for the analysis of Commercial acetaminophen preparations.

Eglal A. Abdelaleem et al.,[21]have developed and validated a simple, sensitive and stability indicating RP-HPLC method for the determination of related substance of Paracetamol and methocarbamol (4-Aminophenol and guaifenesine) in their bulk powders and laboratory prepared mixtures.

Column : ODS column

Mobile phase : 0.05 M KH2PO4 buffer : Acetonitrile (72.5:27.5, v/v, pH = 6)

Flow rate : 1.0mL/min

Wavelength detection : U V detection at 225nm.

The developed method was validated as per ICH guidelines and the calibration plots Were linear over the concentration ranges of 3–20, 4–25, 0.6–8 and 0.6–8 μg mL−1 For Paracetmol, Methocarbamol, 4-Aminophenol and Guafenesine, respectively.

Gnana Raj M et al.,[22] have reported a validated stability indicating RP-HPLC method for the simultaneous estimation of related compounds of ibufrophen and paracetamol in the tablet dosage form.

Column : RP 18 Embedded polar phase column

Mobile phase : Acetonitrile and 0.1 %of orthophosphoric acid (55:45)

Wavelength detection : U V detection at 230nm.

The developed method was validated as per USP and ICH guidelines.

T.A. Phazna Devi1 etal.,[23]have developed High Performance Liquid Chromatography (RP-HPLC) for the determination of paracetamol.

Column	: C_{18} column [4.6x250mm, particle size 5µm].
Buffer	: Ortho phosphoric acid buffer with pH of 3.5.
Mobile phase	: Acetonitrile (ACN) and water (25:75 v/v).
Flow rate	: 1mL/min
Wavelength detection	: UV detector at 207nm

The statistical validation parameters such as linearity, accuracy, precision, interday and intraday variation were checked. The limit of detection and limit of quantification of paracetamol concentrations were found to be 120ng/mL and 360ng/mL. Recovery and assay studies of paracetamol were within 99 to 102% and it was shown that the proposed method could be adoptable for quality control analysis of paracetamol.

Sadana Gangishettyet al.,[24]were described a simple, rapid and reproducible reverse Phasehigh performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of clarithromycin (CLA) and paracetamol (PCM).

Column	: C_{18} column (Kromasil ODS, 5µm, 250 × 4.6mm)
Mobile phase	: Phosphate buffer (0.05M) along with 1-octane sulphonic acid sodium salt monohydrate (0.005M) adjusted to pH 3.2: acetonitrile (50:50).
Flow rate	: 1mL/min
Wavelength detection	: UV detector at 205nm

The developed method was validated according to ICH guidelines Q2 (R1) and found to be linear within the range of 75–175µg/mL for both drug.

Arunadevi S. Birajdar* et al.,[25] have developed high-performance liquid Chromatographic method for the simultaneous estimation of paracetamol and tramadol in combined solid dosage form.

Column	: C_{18} reverse-phase column (250 X 4.6 mm I.D., particle size 5µm).
Buffer	: Triethylamine buffer (pH 7.3)
Mobile phase	: Acetonitrile- 0.26 % Triethylamine buffer (pH 7.3) in ratio of (45:55 % v/v)
Flow rate	: 1mL/min
Wavelength detection	: UV detector at 264nm

The method was linear over the concentration range of 1.0-12.0 µg/mL for paracetmol and 0.1-1.2 µg/mL for tramadol. Domperidone was used as an internal standard (IS).

The analytical recovery obtained was 99.88%. The validation of method carried out as per ICH guidelines.

Swapnalee et al.,[26]have developed simple, selective, rapid, précised and economical RP-HPLC method for the determination of Acetaminophen Caffeine, Phenylephrine Hydrochloride and Dextromethorphan Hydrobromide in the tablet formulation.

Column	: Inertsil C_8 (4.6 mm x 15 cm, 5 µm)
Buffer	: Sodium salt of heptane sulphonic acid buffer solution
Mobile phase	: Sodium salt of heptane sulphonic acid buffer solution and Acetonitrile
Flow rate	: 1mL/min
Wavelength detection	: UV detector at 214nm

Fuad Al-Rimawi*[27] had developed simple, précised and accurate validated HPLC method for the analysis of pseudophedrine hydrochloride, dextromethorphan hydrobromide, chlorpheniramine maleate, and paracetamol in tablet formulations.

Column	: Silica column (5 µm, 125 × 4.6 mm inner diameter)
Buffer	: Ammonium dihydrogen phosphate buffer
Mobile phase	: Methanol: Ammonium dihydrogen phosphate buffer (90:10, v/v)
Flow rate	: 1mL/min
Wavelength detection	: UV detector at 220nm

This new method was validated in accordance with USP requirements for assay accuracy, precision, selectivity, linearity and range, robustness and ruggedness.

I.M. Palabıyık et al.,[28] have developed and validated HPLC method for the simultaneous determination of phenylephrine hydrochloride, paracetamol, chlorpheniramine maleate and dextromethorphan hydrobromide in pharmaceutical preparations.

Column	: C_{18} column
Mobile phase	: Acetonitrile–Sodium perchlorate (0.01M, pH 3)
Flow rate	: 1.4mL/min
Wavelength detection	: UV detector at 204nm

Thummala V. et.al.,[29] have developed sensitive, stability indicating gradient RP-HPLC method for the simultaneous estimation of impurities of Guaifenesin and Dextromethorphan in pharmaceutical formulations.

Column : Sunfire C_{18}, 250cm×4.6mm, 5µm column

Mobile phase : Gradient mixture of solvents A and B

Flow rate : 0.8mL/min

Wavelength detection : UV detector at 224nm

The developed method was validated according to ICH guidelines with respect to specificity, Linearity, limits of detection and quantification, accuracy, precision and robustness. Regression analysis showed an r^2 value (correlation coefficient) greater than 0.999 for Guaifenesin, Dextromethorphan and the impurities.

A. Marín et al.,[30]have developed and validated a RP-HPLC method for the simultaneous estimation of acetaminophen, phenylephrine and chlorphenaramine in pharmaceutical dosage form.

Mobile phase : Mobile phase A : Mobile phase B (92:8)

Mobile phase A : Phosphate buffer 40mM at pH 6.0

Mobile phase B : Acetonitrile

Wavelength detection : UV detector 215nm for phenylephrine and chlopheniramine acetaminophen at 280nm

Gradient program : 75% Mobile Phase 25% Mobile phase B for 8 minutes

30% Mobile phase A 70% Mobile Phase B at 5minute

Rouhollah Heydari[ab*[31]] was reported validated HPLC method for the simultaneous estimation of acetaminophen, phenylephrine, dextromethorphan and chlorpheniramine in pharmaceutical formulation

Column	: 25 cm underivatized silica column
Mobile phase	: Methanol: water (containing 6.0 g of ammonium acetate and 10 ml of triethylamine per liter, pH adjusted to 5.0 with orthophosphoric acid), 95:5%(v/v)
Wavelength detection	: UV-vis detector at 254 nm for acetaminophen at 220 nm for phenylephrine, and at 227 nm for dextromethorphan and chlorpheniramine.

The method showed linearity for the acetaminophen, phenylephrine, dextromethorphan, and chlorpheniramine in the 162.5–650, 2.5–10, 7.5–30, and 1–4 µg/ml ranges, respectively. The intraday and interday RSDs ranged from 0.92 to 1.52%, 1.00 to 1.76%, 1.21 to 1.74% and 1.26 to 1.80% for the acetaminophen, phenylephrine, dextromethorphan, and chlorpheniramine, respectively.

Marín et al.,[32]have developed HPLC method for the simultaneous determination of acetaminophen, phenylephrine and chlorpheniramine in capsules as pharmaceutical formulation after their dissolution test.

Column	: HS PEG column (polyethylene glycol), 5 µm,
Mobile phase	: 20 mM phosphate buffer at pH 7.0/acetonitrile 80:20 (v/v).
Flow rate	: 1mL/min
Wavelength detection	: UV detection at 210 nm for phenylephrine and chlorpheniramine and at 305 nm for acetaminophen.

The validation parameters of both techniques were adequate for the intended purpose.

JOSHI et al.,[33]have developed and validated isocratic RP-HPLC method for the simultaneous estimation of dextromethorphan hydrobromide and levocetirizine dihydrochloride in a cough syrup. The method was validated as per ICH guidelines.

Column	: Phenomenex (USA) C18 analytical column, 250x4.0 mm
Mobile phase	: potassium dihydrogen phosphate buffer (pH 2.5) – acetonitrile and tetrahydrofuran (70:25:5, v/v/v).
Flow rate	: 1.2mL/min
Wavelength detection	: U V detection at 232nm

Brahmbhatt* et al.,[34]have reported simple, accurate and precised RP-HPLC method for Tramadolhydrochloride (THC) and Paracetamol (PCM).

Column	: C_{18} ODS column (150 × 4.6 mm)
Mobile phase	: Water : Methanol (70:30 v/v) at pH 3.4 adjusted by 5% o-phosphoric acid
Flow rate	: 1.0mL/min
Wavelength detection	: U V detection at 228nm.

Linearity was observed over the concentration range of 195-455 µg/ml and 22.5-52.5 µg/ml for PCM and THC respectively. The LOD was found to be 6.999 and 0.80 for PCM and THC respectively. LOQ was found to be 2.10 for PCM and 2.42 for THC. Moreover, the % RSD for repeatability, inter and intraday precision was found to be less than 2%, which reveals that the method is precise. The % recovery was found to be 99.83%- 100.30%. For PCM and 98.52- 101.64% for THC.

4. AIM AND PLAN OF WORK

Aim and scope

Acetaminophen, Dextromethorphan HBr and Phenylephrine HCl are official in U.S.P. From the literature survey, it was found that there were only few RP-HPLC methods reported for the estimation of related substances of Acetaminophen in tablet formulation and no analytical method was reported for the estimation of related substances of acetaminophen in capsule formulation.

Hence, the aim of present work is to develop RP-HPLC method for estimation of related substances of Acetaminophen in acetaminophen, phenylephrine Hcl and dextromethorphan HBr soft gelatin capsules and to validate the developed RP-HPLC method by validation parameters as per ICH guidelines.

Plan of present work

- Method development (By reverse phase HPLC).
- Validation of the developed method (By using following parameters).
    a. System suitability studies
    b. Limit of Detection
    c. Limit of Quantification
    d. Linearity and Range
    e. Accuracy
    f. Precision
    g. Specificity
    h. Robustness
    i. Ruggedness

5. MATERIALS AND INSTRUMENT USED

a) Active Ingredients used:

S.No.	Name	Specification
1.	Acetaminophen	As Referance standard
2.	4-Aminophenol	As Referance standard
3.	4-Chloroacetanilide	As Referance standard
4.	Dextromethorphan HBr	As Referance standard
5.	Phenylephrine Hcl	As Referance standard

b) Apparatus / Instruments used:

S.No.	NAME	MODEL	MANFACTURER/SUPPLIER
1.	Weighing balance	AUM220D	Schimadzu
2.	Sonicator	sonorex	Sonorex dg 10p
3.	pH Meter	9087	ELICO pH METER
4.	HPLC-UV,PDA	LC-2010	Schimadzu
5.	Colum	Zodiac C18	Zodiac

C) Chemical used:

S.No.	NAME	GRADE	MANFACTURER/SUPPLIER
1.	Pottasium di hydrogen orthophosphate	HPLC	Rankem Chemicals
2.	1-pentane sulponic acid sodium salt	HPLC	Rankem Chemicals
3.	Acetonitrile	HPLC	Afladam Chemicals
4.	Methonol	HPLC	Rankem Chemicals
5.	Phosphoric acid	HPLC	Merck Chemicals
6.	Tri Ethylamine	HPLC	Rankem Chemicals
7.	Milli-Q Wter	HPLC	In House production

6. RP-HPLC METHOD DEVELOPMENT

The method development stage, decision regarding choice of column, mobile phase, detectors and method of quantitation must be addressed. In this way, development considers all the parameters pertaining to any methods [15].

(1)Selection of stationary phase

Proper selection of the stationary phase depends up on the nature of the sample and chemical profile. The drug selected for the present study was polar compound and could be separated either by normal phase chromatography or reverse phase chromatography. From literature survey, it was found that different C_{18}columns could be appropriately used for the separation of related substances for Acetaminophen.

(2)Selection of wavelength

The sensitivity of the HPLC method depends upon the selection of detection wavelength. An ideal wavelength is one that gives good response for related substances and the drugs to be detected. The wavelength for measurement was selected as 245 nm from the absorption spectrum.

(3)Selection of mobile phase

The mobile phase was selected and chromatograms were recorded, trails were done on different mobile phases.

TRIALS

Trial-1

Buffer preparation:

6.8 g of Potassium dihydrogen orthophosphate was dissolved in 1000 mL of milli-Q water and pH was adjusted to 3.0 with orthophosphoric acid. Then it was filtered through 0.45 µ nylon membrane filter and degassed.

Mobile phase A: Buffer (70%)

Mobile phase B: Methanol(30%)

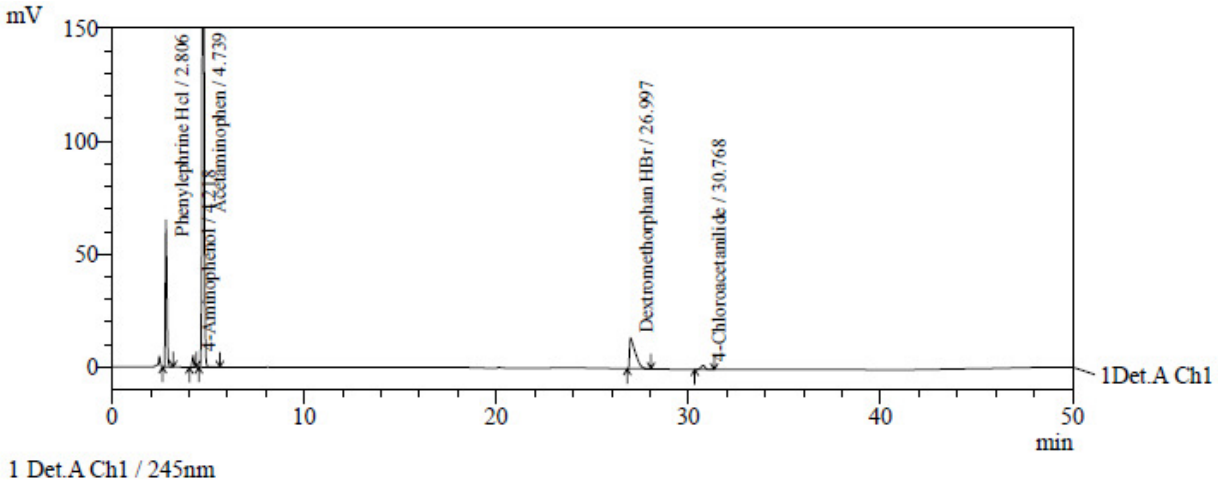
Chromatographic conditions

Column	: Zodiac C-18, (250 × 4.6 mm, 5 µ)
Detector wavelength	: 245 nm
Column temperature	: 30 ºC
Injection Volume	: 10 µL
Flow rate	: 1.0 mL/min
Runtime	: 55 min

Observation:

Resolution of peaks were not satisfactory for 4-Aminophenol, Phenylephrine HCl and Acetaminophen and Dextromethorphan HBr.

Figure-13



Trial 2:

Buffer Preparation:

2.0 g of Sodium di hydrogen orthophosphate was dissolved in 1000 mL of milli-Q water and pH was adjusted to 3.0 with orthophosphoric acid. Then it was filtered through 0.45 µ nylon membrane filter and degassed.

Mobile phase A: Buffer (70%)

Mobile phase B: Acetonitrile(30%)

Chromatographic Condition:

Column & ID NO : Zodiac C-18, (250 × 4.6 mm, 5 µ)

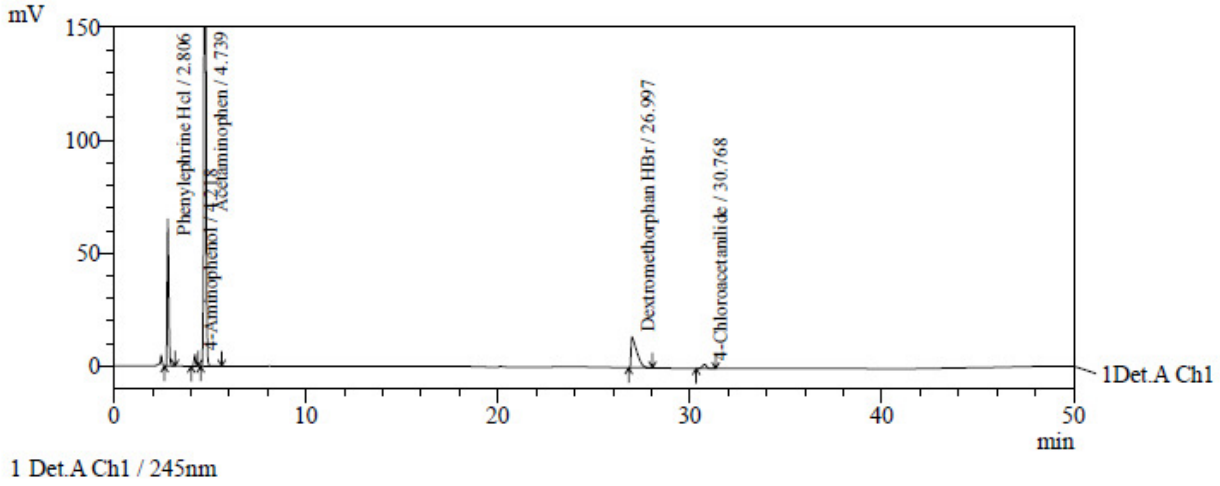
Column temperature : 30°C

Inj. Volume : 10 µL

Flow rate : 1.0ml/min

λ_{max} : 245 nm

Figure-14



Observation:

Resolution of peaks were not satisfactory for 4-Aminophenol, Phenylephrine HCl and Acetaminophen and Dextromethorphan HBr

Trial 3: (Optimized Method)

Buffer preparation – 1

1.92g of 1-Pentane sulphonic acid sodium salt was dissolved in 1000ml distilled water followed by the addition of 0.1ml Triethylamine and pH was adjusted to 6.0 with Orthophosphoric acid.

Buffer preparation - 2:

6.8g of potassium Dihydrogen ortho phosphate was dissolvedin1000ml distilled water and pH was adjusted to 3.0 with orthophosphoric acid.

Mobile phase A: Buffer-1: Methanol (85:15)

Mobile phase B: Buffer-2: Acetonitrile (70:30)

Chromatographic conditions

Column : Zodiac C-18, (250 × 4.6 mm, 5 µ)

Detector wavelength : 245 nm

Column temperature : 30 ºC

Injection Volume : 20 µL

Flow rate : 1.0 mL/min

Runtime : 55 min

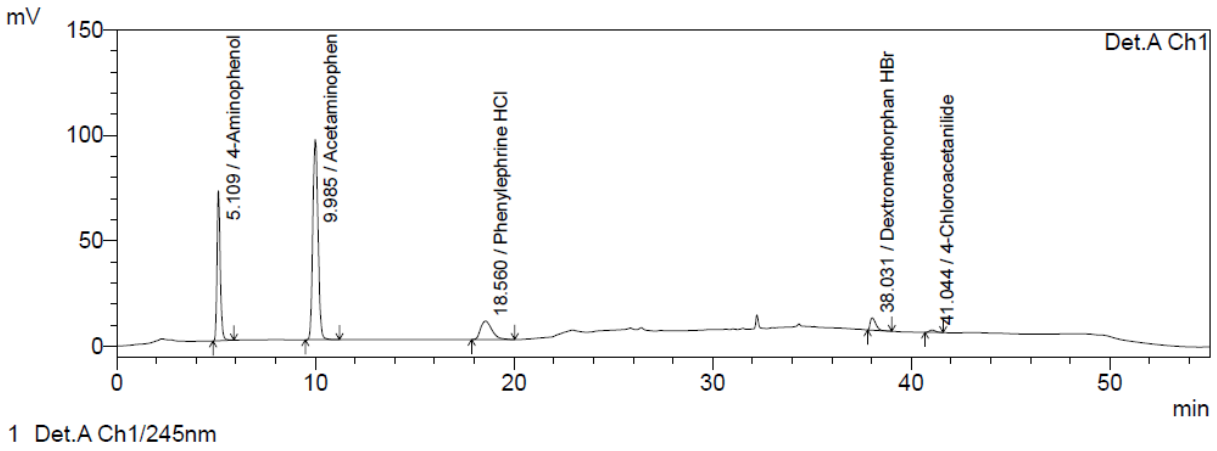
Diluent **:** Mobile phase-A

Gradient Program:

Table No: 2

Time (mins)	Mobile phase-A (%)	Mobile phase-B (%)
0.01	100	0
15.0	100	0
30.0	0	100
45.0	0	100
50.0	100	0
55.0	100	0

Figure-15



1 Det.A Ch1/245nm

Detector A Ch1 245nm

Peak#	Name	Ret. Time	Area	Area %	Tailing Factor	Theoretical Plates	Resolution
1	4-Aminophenol	5.11	802972	26.52	1.3	4671	0.0
2	Acetaminophen	9.98	1747425	57.72	1.1	6752	12.4
3	Phenylephrine HCl	18.56	340689	11.25	1.4	5401	11.5
4	Dextromethorphan HBr	38.03	113584	3.75	1.7	88869	25.6
5	4-Chloroacetanilide	41.04	22726	0.75	1.2	83527	5.6

Observation:

Resolution was satisfactory for 4-Aminophenol, Phenylephrine HCl and Acetaminophen and Dextromethorphan HBr.

Sample preparation

Preparation of sample solution:

8000mg of medicament was accurately weighed (equivalent to 2000mg of Acetaminophen) and transfered into a 100 ml volumetric flask.70mL of diluent was added and sonicated for 30minutes. Finally volume was made up with diluents and Filtered the solution through whatman filter no:42.

Preparation of Diluted Standard Solutions:

Acetaminophen:

50mg of Acetaminophen working standard was accurately weighed and transfered into a 50 ml Volumetric flask.30ml of diluent was added and sonicated and volume was made up with diluent. 5mlof stock solution was pipetted out into a 50 ml volumetric flask and finally volume was made up with diluent (Solution 1).

4-Chloroacetanilide:

20mg of 4-Chloroacetanilide working standard was accurately weighed and transferred in to a 50 ml Volumetric flask. 30ml of diluent was added and sonicated. Then volume was made up with diluent.5ml of stock solution was pipetted out into a 50 ml volumetric flask and volume was made up with diluent. 5ml of resulting solution was pipetted out into a 100ml of volumetric flask and finally volume was made up with diluent (Solution 2).

4-aminophenol:

50mg of 4-Aminophenol working standard was accurately weighed and transfered into a 50 ml Volumetric flask .30ml of diluent was added and sonicated . Then volume was made up with diluent.10ml of stock solution was pipetted out into a 50 ml volumetric flask and finally volume was made up with diluent (Solution 3).

Resolution solution: (Diluted standard)

20mg of Phenylephrine HCl WS/RS and 10mg of Dextromethorphan HBr WS/RS were accurately weighed and transfered into a 50ml volumetric flask. 10ml of Solution 1, 5ml of Solution 2 and 5ml of Solution 3, were added into a 50ml volumetric flask and finally volume was made up with diluent.

7. METHOD VALIDATION

VALIDATION

According to ICH guidelines method validation can be defined as "Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics" [18]. Such validated analytical method for qualitative and quantitative testing of the drug molecule assume greater importance when they are employed to generate quality and safety compliance data during development, pre-formulation studies and post approval of drug products.

The ICH of Technical Requirements for the Registration of Pharmaceutical for human use has developed a consensus text on the validation of analytical procedures. The document includes definitions for eight validation characteristics

Parameters Used for Assay Validation

The validation of the assay procedure was carried out using the following parameters.

1) **Parameters:**

1.1 System suitability

1.2 Specificity

1.3 Limit of Detection
1.4 Limit of Quantification
1.5 Linearity and Range
1.6 Accuracy
1.7 Precision
1.8 Robustness

Reagents:

Buffer preparation – 1

1.92g of 1-Pentane sulphonic acid sodium salt was dissolved in 1000ml distilled Water followed by the addition of 0.1ml Triethylamine and pH was adjusted to 6.0 with Orthophosphoric acid.

Buffer preparation - 2:

6.8g of potassium Dihydrogen orthophosphate was dissolved in1000ml distilled water and pH was adjusted to 3.0 with orthophosphoric acid.

Mobile Phase:

Mobile Phase – A:

850ml of Buffer-1 and 150ml of Methanol were mixed. Then solution was filtered through 0.45µm nylon membrane filter and it was degassed.

Mobile Phase – B:

700ml of Buffer-2 and 300ml of Acetonitrile were mixed. Then solution was filtered through 0.45µm nylon membrane filter and it was degassed.

Diluent: Mobile phase – A

Gradient Program:

Table No:2

Time (mins)	Mobile phase-A (%)	Mobile phase-B (%)
0.01	100	0
15.0	100	0
30.0	0	100
45.0	0	100
50.0	100	0
55.0	100	0

7.1 PREPARATION OF STANDARD AND SAMPLE SOLUTION:

STANDARD PREPARATION:

Acetaminophen:

50mg of Acetaminophen working standard was accurately weighed and transfered into a 50 ml Volumetric flask, 30ml of diluent was added and sonicated. Then volume was made up with diluent. 5ml of stock solution was pipetted out into a 50 ml volumetric flask and finally volume was made up with diluent (Solution 1).

4-Chloroacetanilide:

20mg of 4-Chloroacetanilide working standard was accurately weighed and transferred in to a 50 ml Volumetric flask. 30ml of diluent was added and sonicated. Then volume was made up with diluent. 5ml of stock solution was pipetted out into a 50 ml volumetric flask and volume was made up with diluent. 5ml of resulting solution was pipetted out into a 100ml of volumetric flask and finally volume was made up with diluent (Solution 2).

4-aminophenol:

50mg of 4-Aminophenol working standard was accurately weighed and transfered in to a 50 ml Volumetric flask. 30ml of diluent was added and sonicated. Then volume was made up with diluent. 10ml of stock solution was pipetted out into a 50 ml volumetric flask and finally volume was made up with diluent (Solution 3).

Resolution solution: (Diluted standard)

20mg of Phenylephrine HCl WS/RS and 10mg of Dextromethorphan HBr WS/RS were accurately weighed and transfered into a 50ml volumetric flask. 10ml of Solution 1, 5ml of Solution 2, and 5ml of Solution 3 were added into a 50ml volumetric flask and finally volume was made up with diluent.

Sample Preparation:

8000mg of medicament was accurately weighed (equivalent to 2000mg of Acetaminophen) and transfered into a 100 ml volumetric flask . 70mL of diluent was added and sonicated for 30minutes. Finally volume was made up with diluent and Filtered the solution through whatman filter no: 42.

Spiked Sample Preparation:

8000mg of medicament was accurately weighed (equivalent to 2000mg of Acetaminophen) and transfered into a 100 ml volumetric flask. 10ml of Solution-2, 10ml of Solution-3 and

70mL of diluent were added and sonicated for 30minutes. Finally volume was made up with diluent and Filtered the solution through whatman filter no: 42.

7.2 CHROMATOGRAPHIC PARAMETERS:

The liquid chromatographic system was set as follows:

Table No: 3

Instrument	**HPLC with UV/PDA detector**
Column	C_{18} – 250 mm x 4.6 mm, 5m (zodiac or equivalent)
Flow rate	1.0 ml/min.
Oven temperature	30.0°C
Sample temperature	Ambient
Diluent	Mobile phase
Wavelength	245 nm
Injection volume	20 µl

Calculation:

Calculation of impurities:

(1) 4- Aminophenol =

$$\frac{AKIT1}{ADS1} \times \frac{WS1}{50} \times \frac{10}{50} \times \frac{5}{50} \times \frac{100}{WT} \times \frac{P1}{100} \times \frac{\text{Avg.net weight in mg}}{LCA} \times 100$$

(2) 4- Chloroacetanilide =

$$\frac{AKIT2}{ADS2} \times \frac{WS2}{50} \times \frac{5}{50} \times \frac{5}{100} \times \frac{5}{50} \times \frac{100}{WT} \times \frac{P2}{100} \times \frac{\text{Avg.net weight in mg}}{LCA} \times 100$$

(3) Total Unknown impurities =

$$\frac{ATIT1}{ADS} \times \frac{WS3}{50} \times \frac{5}{50} \times \frac{10}{50} \times \frac{100}{WT} \times \frac{P3}{100} \times \frac{\text{Avg.net weight in mg}}{LCA} \times 100$$

AKIT1 = Area of 4-Aminophenol impurity obtained from test preparation.
AKIT2 = Area of 4-Chloroacetanilide impurity obtained from test preparation.
ATIT1 = Sum of all peak area in test solution – [Test solution Known impurity peak area + Acetaminophen peak area]
ADS = Area of Acetaminophen peak obtained from Resolution solution.
ADS1 = Area of 4- Aminophenol peak obtained from Resolution solution.

ADS2 = Area of 4- Chloroacetanilide peak obtained from Resolution solution.
WS1 = Weight of 4- Aminophenol working standard taken in mg.
WS2 = Weight of 4-Chloroacetanilide working standard taken in mg.
WS3 = Weight of Acetaminophen working standard taken in mg.
WT = Weight of test sample taken in mg for test preparation.
P1 = Purity of 4- Aminophenol working standard as such basis in %.
P2 = Purity of 4-Chloroacetanilide working standard as such basis in %.
P3 = Purity of Acetaminophen working standard as such basis in %.
LCA = Label claim of Acetaminophen.

Acceptance Criteria:

1. 4-Chloroacetanilide: NMT 0.001
2. 4-aminophenol: NMT 0.1%
3. Total Unknown impurities: NMT 0.1%

SYSTEM SUITABILITY

System suitability parameters and recommendations were shown in the table no.4

Table no. 4 System suitability parameters and recommendations

S.No	Parameters	Recommendations
1	Theoretical plates (N)	>2000
2	Tailing factor (T)	≤ 2
3	Resolution (Rs)	> 2 between peak of interest and the closest eluting potential interference
4	Repeatability	RSD ≤ 2% for N ≥5 is desirable
5	Capacity factor (k^1)	> 2.0
6	Relative retention	Not essential as long as the resolution is stated

Procedure:

A standard solution (resolution solution) was injected .Six times into HPLC system and system suitability parameters were evaluated. Chromatograms were shown in the figure-16 and data was shown in the table no.5, 6and 7.

Figure -16 Chromatogram for system suitability

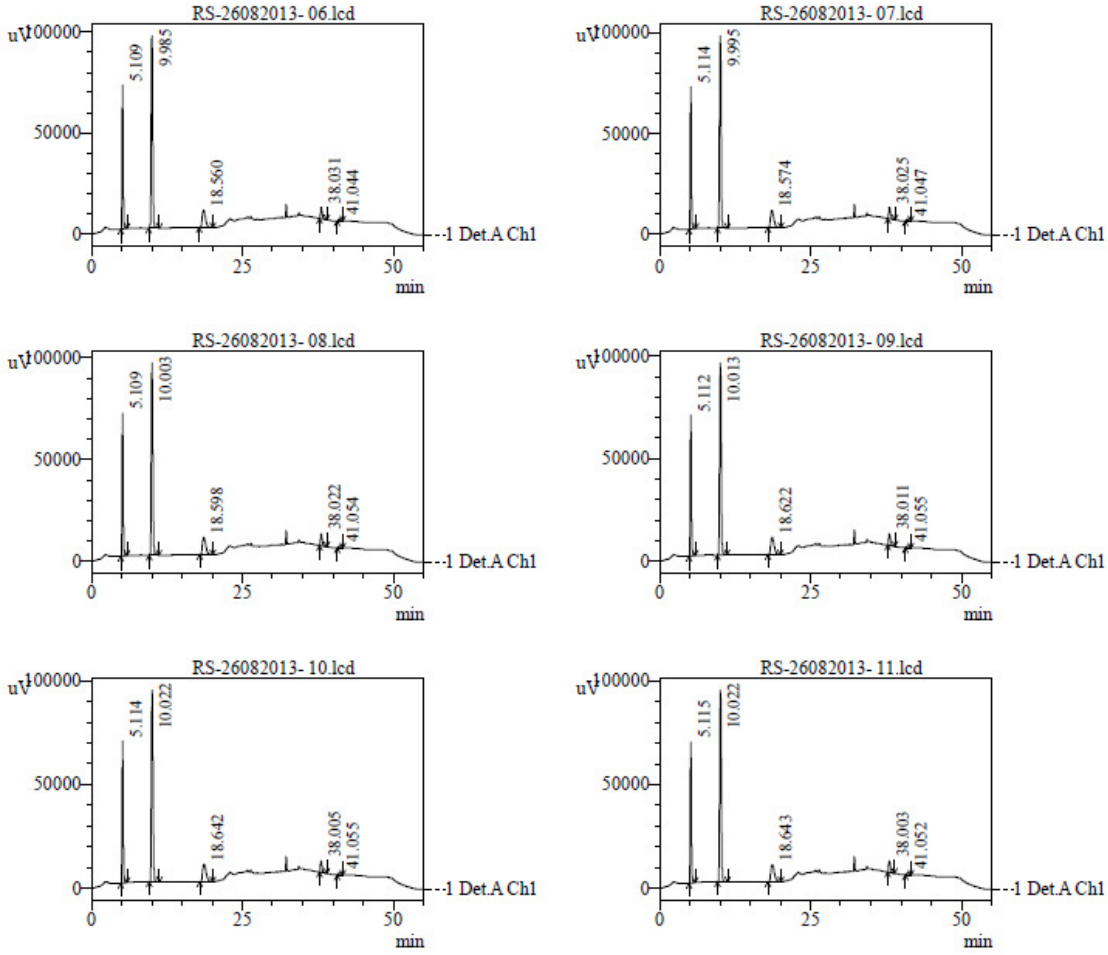


Table No: 5 Data for system suitability (4-Aminophenol)

No of Injection	RT	Theoretical plate	Tailing factor	Peak response
01	5.11	4671	1.3	692972
02	5.11	4517	1.3	692924
03	5.11	4503	1.3	690825

04	**5.11**	**4301**	**1.3**	**698031**
05	5.11	4372	1.3	696874
06	5.12	4306	1.3	694740
Mean	**5.11**	**4445**	**1.3**	**694394**
SD	**0.004**	**144.9**	**0.0**	**2698.8**
%RSD	**0.1**	**3.3**	**0.0**	**0.4**

Table No: 6 Data for system suitability (Acetaminophen)

No of Injection	RT	Theoretical plate	Tailing factor	Peak response
01	9.98	6752	1.1	1747425
02	9.99	6767	1.1	1757528
03	10.00	6671	1.2	1747334
04	10.01	6551	1.2	1746367
05	10.02	6430	1.2	1741372
06	10.02	6331	1.2	1754869
Mean	**10.00**	**6584**	**1.2**	**1749149**
SD	**0.0**	**177.8**	**0.1**	**5956.7**
%RSD	**0.2**	**2.7**	**4.4**	**0.3**

Table No: 7 Data for system suitability (4-Chloroacetanilide)

No of Injection	RT	Theoretical plate	Tailing factor	Peak response
01	41.04	83527	1.2	24726
02	41.05	82925	1.2	24627
03	41.05	81857	1.2	24491
04	41.06	81225	1.2	24435
05	41.06	80478	1.2	24172
06	41.05	79680	1.2	24230
Mean	**41.05**	**81615**	**1.2**	**24447**
SD	**0.0**	**1457.4**	**0.0**	**216.8**
%RSD	**0.0**	**1.8**	**0.0**	**0.9**

SPECIFICITY

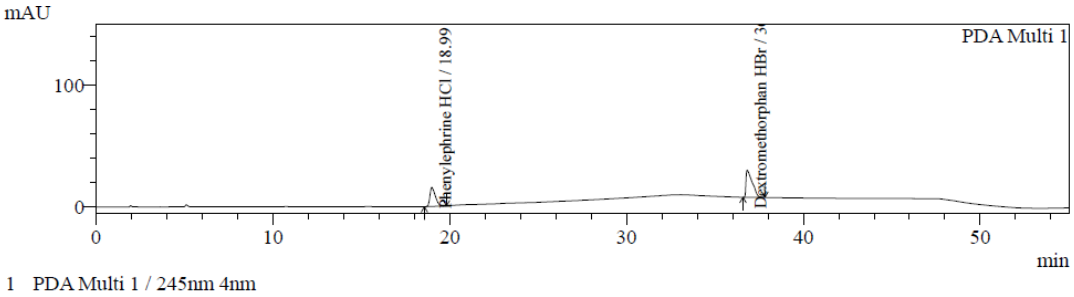
Blank, standard, placebo, all known related compounds, spiked sample, sample solutions were prepared and injected into the chromatographic system for identification and interference with the Acetaminophen, Phenylephrine HCl and Dextromethorphan HBr peaks.

a. Placebo Interference:

A study to establish the interference of placebo was conducted. Sample preparation of

placebo was done as that of test sample preparation of assay method. Chromatogram of placebo did not show any additional peaks. This indicated that the excipients used in the formulation did not interfere in the RS of Acetaminophen, Phenylephrine HCl and Dextromethorphan HBr Capsules. Resulted chromatograms were shown in the figure-17,19,20 and data was shown in the table no 8 and 9.

Figure-17 Chromatogram for placebo



BLANK INTERFERENCE:

A study to establish the interference of blank was conducted. Diluent was injected as per the test method. Chromatogram was appended in figure no.18 and data was shown in table no. 8 and 9.

Figure-18 Chromatogram of blank

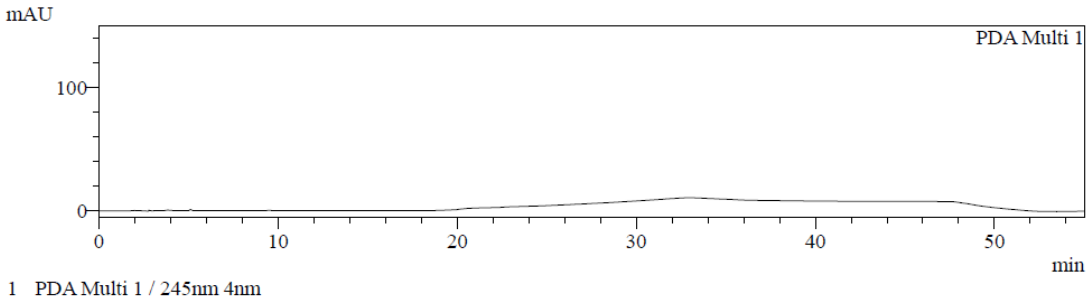
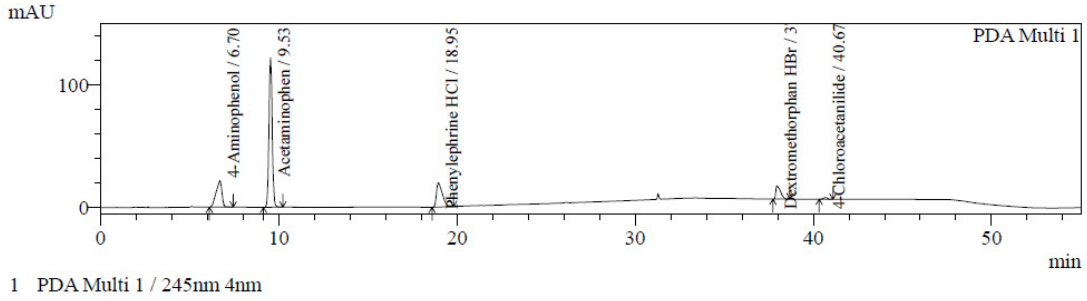
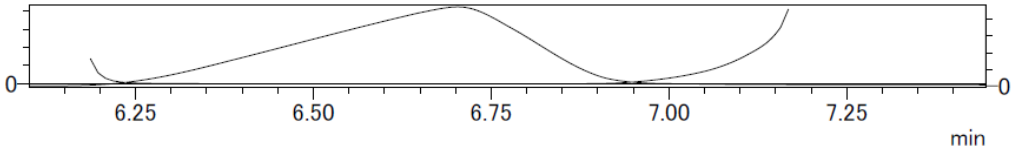


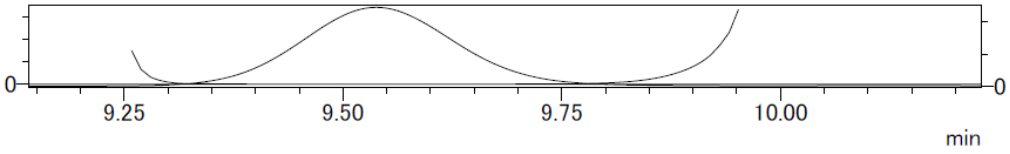
Figure-19 Chromatogram of standard



ID# : 1
Retention Time : 6.697
Compound Name : 4-Aminophenol



ID# : 2
Retention Time : 9.533
Compound Name : Acetaminophen



ID# : 5
Retention Time : 40.671
Compound Name : 4-Chloroacetanilide

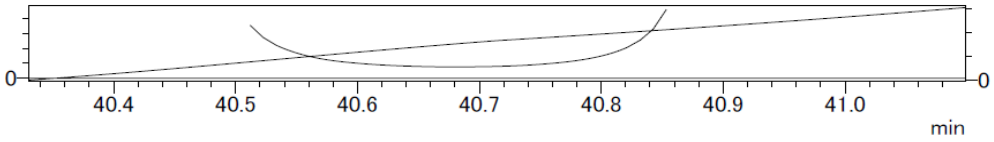
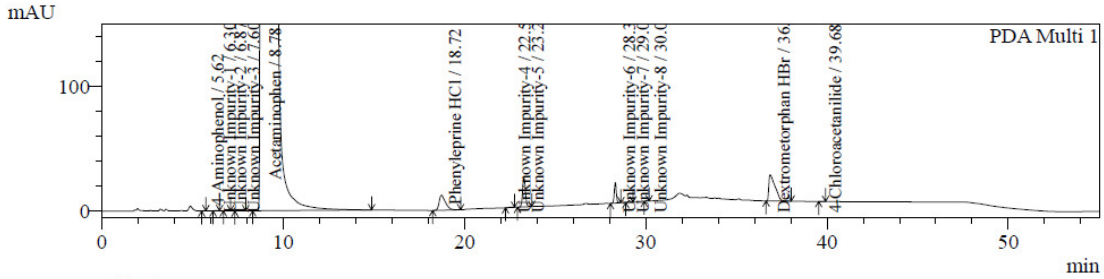
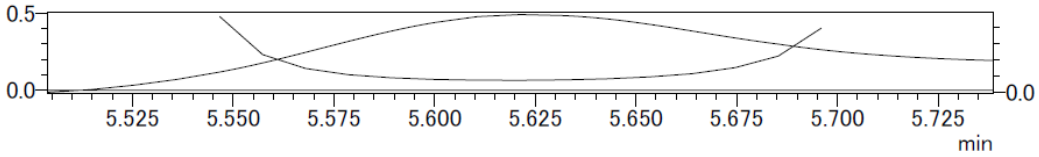


Figure-20 Chromatogram of Sample

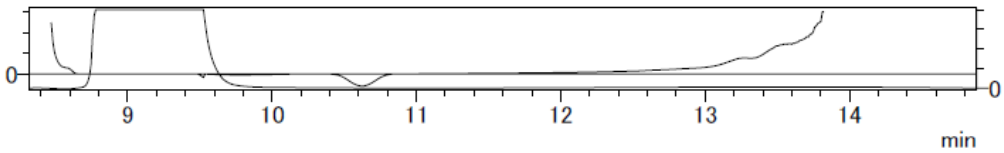


1 PDA Multi 1 / 245nm 4nm

ID# : 1
Retention Time : 5.618
Compound Name : 4-Aminophenol



ID# : 5
Retention Time : 8.779
Compound Name : Acetaminophen



ID# : 13
Retention Time : 39.681
Compound Name : 4-Chloroacetanilide

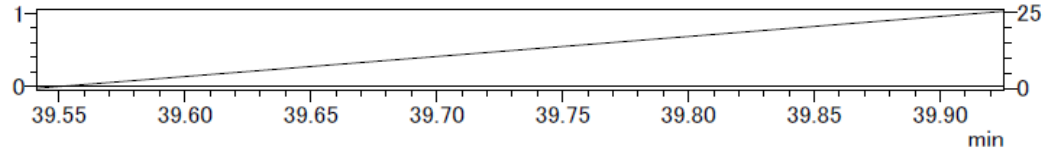


Table No: 8 Data for placebo and blank interference

	RT	Peak response	Peak Purity index
Blank	NA	NA	NA
Placebo	NA	NA	NA
Working standard			
4-Aminophenol	6.70	682115	1.000
Acetaminophen	9.53	1631649	1.000
4-Chloroacetanilide	40.67	29851	0.999
Sample with spike			
4-Aminophenol	6.50	693280	0.999
Acetaminophen	8.77	201805492	0.999
4-Chloroacetanilide	39.69	30457	1.000

Acceptance criteria:

No interference was found at the retention times of Acminophen, 4-Aminophenol and 4-Chloroacetanilide.

Table No: 9 Interference of blank, placebo and impurity

Name	Interference	Retention time (minutes)
Blank	Nil	Nil
Placebo	Nil	Nil

Precision:

Method Precision:

Six spiked sample preparations were prepared individually using single batch of Acetaminophen, Phenylephrine HCl and Dextromethorphan HBr Capsules as per test method and injected each solution. Resulted chromatogram was shown in the figure-21. and data was shown in the Table no.10 & 11.

Figure No: 21 Chromatograms for sample

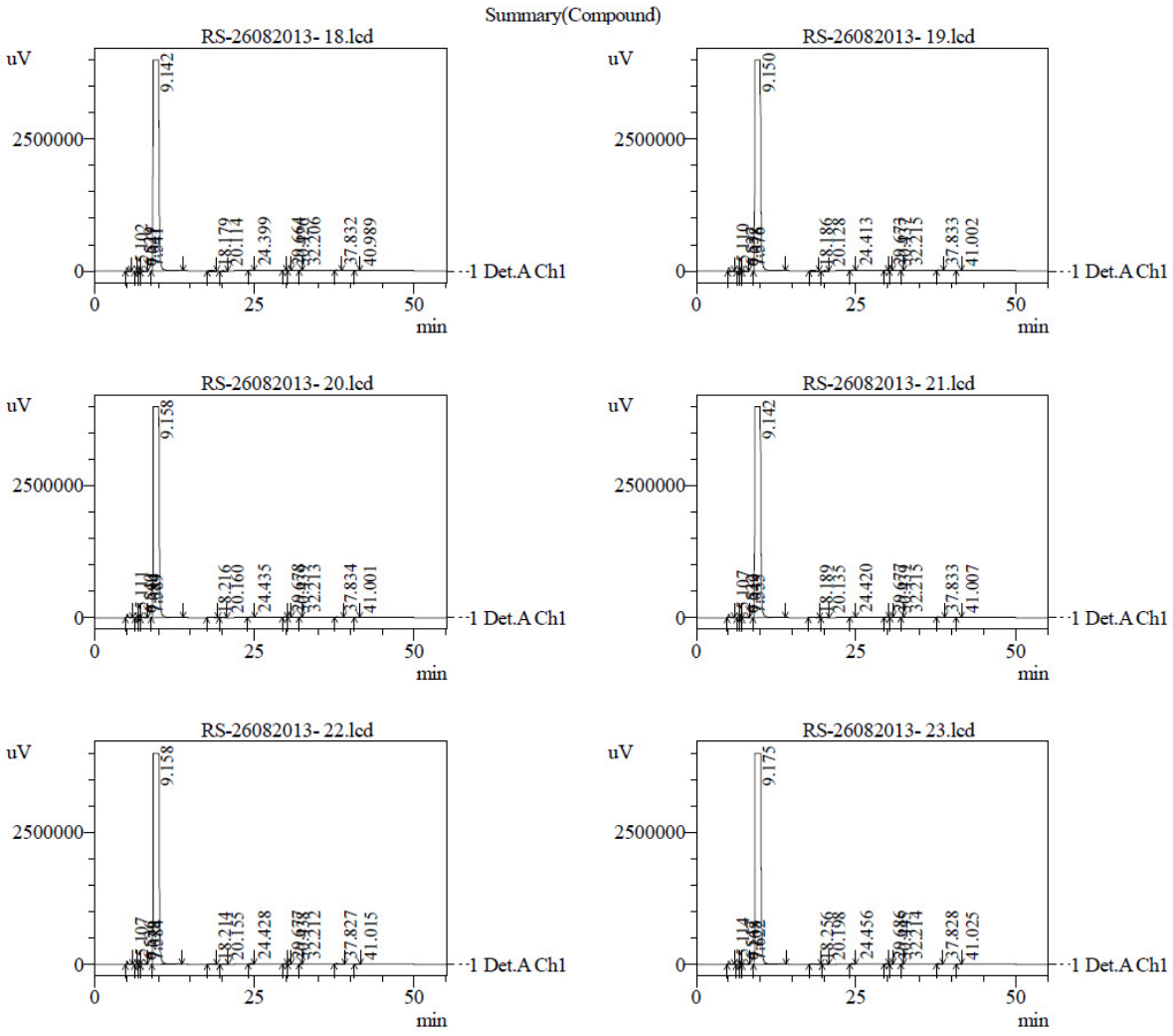


Table No: 10 Data for repeatability

No. of Sample	4-aminophenol	4-Chloroacetanilide	Total Impurity
	%		
01	103.2	101.7	0.03
02	101.9	105.8	0.04
03	102.5	105.9	0.03
04	104.1	101.3	0.03
05	103.9	104.3	0.04
06	102.4	101.3	0.04
Mean	**103.0**	**103.4**	**0.04**
SD	**0.8**	**2.2**	**0.005**
% RSD	**0.8**	**2.1**	**12.5**
Confidence	0.42	0.56	0.00

Table No: 11 Results of method precision

System Suitability	Results	Acceptanc

Parameters	4-Aminophenol	Acetaminophen	4-Chloroacetanilide	e criteria
Tailing factor for Acetaminophen, 4-Aminophenol and 4-Chloroacetanilide peaks from first standard injection.	1.3	1.1	1.2	**NMT 2.0**
Theoretical plate count for Acetaminophen, 4-Aminophenol and 4-Chloroacetanilide peaks from first standard injection.	4671	6752	83527	**NLT 2000**
The %RSD of RT for six replicate injection of standard solution (for 4-Aminophenol, Acetaminophen and 4-Chloroacetanilide)	0.1	0.2	0.0	**NMT 1.0**
The %RSD of Peak response from six replicate injection of standard solution (for 4-Aminophenol, Acetaminophen and 4-Chloroacetanilide)	0.4	0.3	0.9	**NMT 2.0**
Resolution between 4-Aminophenol and Acetaminophen injection from first standard injection.	12.4		N/A	**NLT 2.0**
% recovery of known impurities in six spiked samples	103.0	N/A	103.4	**90.0-110.0**
% RSD of known impurities in six spiked samples.	0.8	N/A	2.1	**NMT 10.0**

Ruggedness & Intermediate Precision:

Intermediate precision experiment was repeated by a second analyst a different HPLC system and a separate HPLC column on a different day. The second analyst prepared all reagents, sample solutions and standard solutions required for the analysis. Resulted chromatograms were shown in the figure-22&23 and data was shown in the table no.12, 13, 14, 15.

Figure-22 Chromatograms for Ruggedness

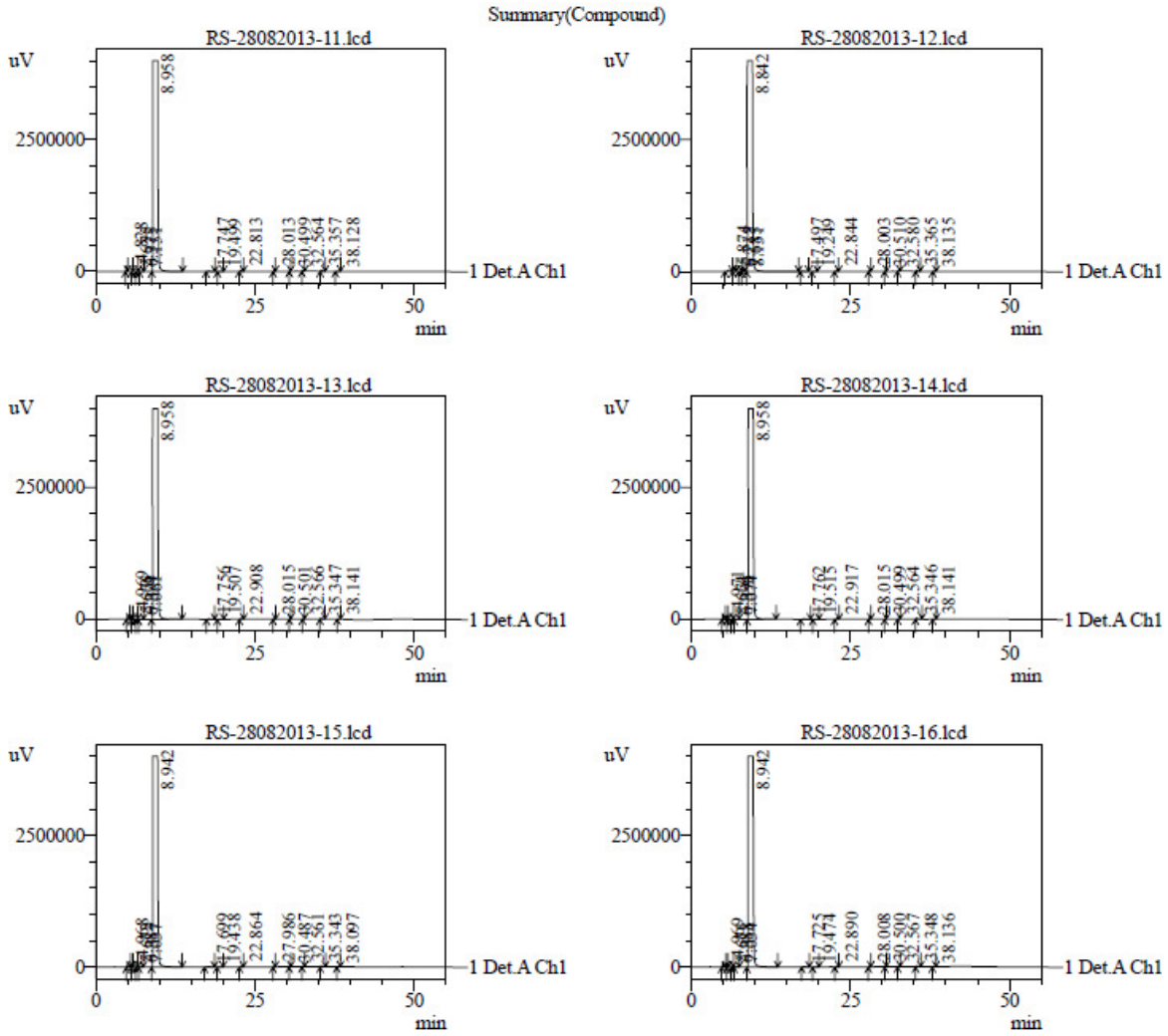


Figure-23 Chromatograms for Intermediate Precision

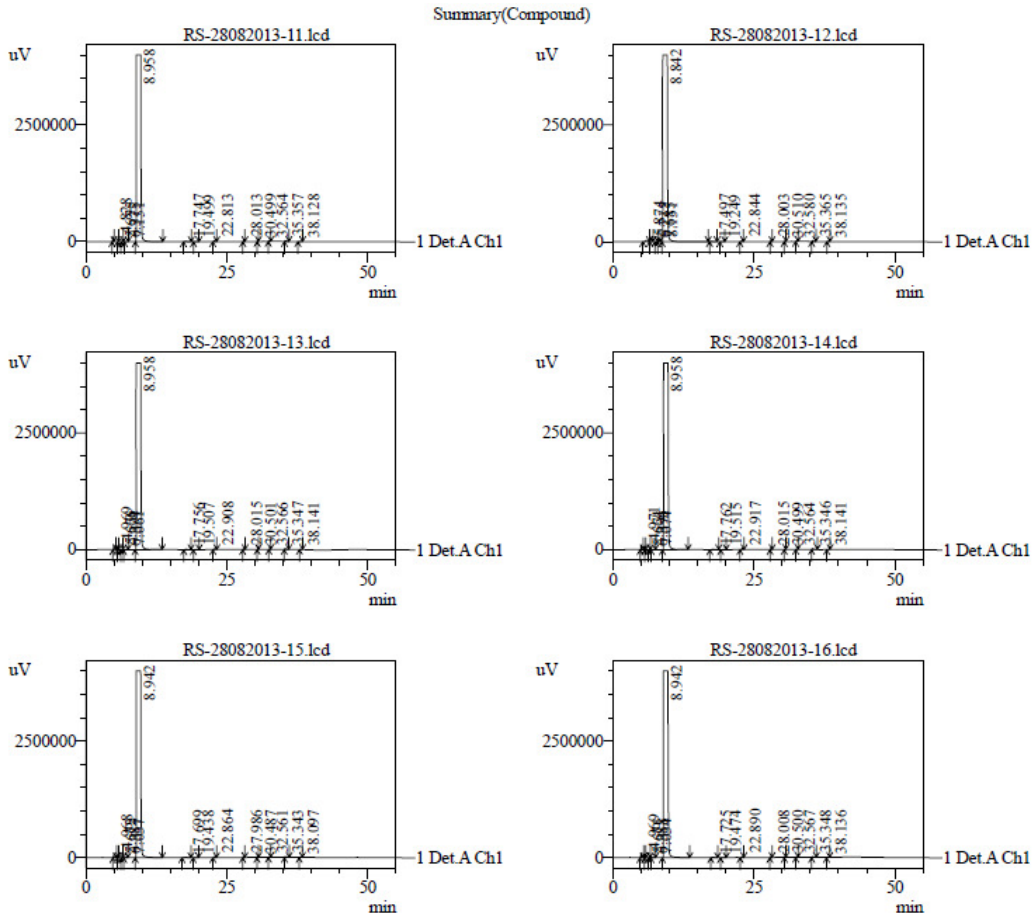


Table No: 12 Data for ruggedness and intermediate precision (4-Aminophenol)

No of Injection	RT	Theoretical plate	Tailing factor	Peak response
01	4.71	9550	1.0	671190
02	4.71	9544	1.0	669650
03	4.71	9569	1.0	669055
04	4.71	9561	1.0	665927
05	4.71	9524	1.0	665060
06	4.72	9508	1.0	662250
Mean	**4.71**	**9543**	**1.0**	**667189**
SD	**0.0**	**23**	**0.0**	**3349**
%RSD	**0.0**	**0.2**	**0.2**	**0.5**

Table No: 13 Data for ruggedness and intermediate precision(Acetaminophen)

No of Injection	RT	Theoretical plate	Tailing factor	Peak response
01	9.27	15655	1.1	1636222
02	9.27	15675	1.1	1635268
03	9.27	15662	1.1	1636067
04	9.29	15643	1.1	1634471
05	9.29	15589	1.1	1634828
06	9.31	15602	1.1	1636635
Mean	**9.28**	**15638**	**1.1**	**1635582**
SD	**0.01**	**35**	**0.0**	**855**
%RSD	**0.2**	**0.2**	**0.1**	**0.1**

Table No: 14 Data for ruggedness and intermediate precision (4- chloroacetanilide)

No of Injection	RT	Theoretical plate	Tailing factor	Peak response
01	37.46	219780	1.0	30864
02	37.46	224300	1.0	30123
03	37.47	223459	1.0	30026
04	37.47	223522	1.0	29997
05	37.48	223599	1.0	29880
06	37.48	223977	1.0	29749
Mean	**37.47**	**223106**	**1.0**	**30106**
SD	**0.01**	**1660**	**0.0**	**393**
%RSD	**0.0**	**0.7**	**0.4**	**1.3**

Table No: 15 Results of ruggedness and intermediate precision

System Suitability Parameters	Results			Acceptance criteria
	4-aminophenol	**Acetaminophen**	**4-Chloroacetanilide**	
Tailing factor for Acetaminophen, 4-Aminophenol and 4-Chloroacetanilide peaks from first standard injection.	1.0	1.1	1.1	**NMT 2.0**
Theoretical plate count for Acetaminophen, 4-Aminophenol and 4-Chloroacetanilide peaks from first standard injection.	9550	15655	219780	**NLT 2000**
The %RSD of RT for six replicate injection of standard solution (for 4-Aminophenol, Acetaminophen and 4-Chloroacetanilide)	0.0	0.2	0.0	**NMT 1.0**
The %RSD of Peak response from six replicate injection of standard solution (for 4-Aminophenol, Acetaminophen and 4-Chloroacetanilide)	0.5	0.1	1.3	**NMT 2.0**
Resolution between 4-Aminophenol and Acetaminophen injection from first standard injection.	18.7		7.4	**NLT 2.0**
Confidence limits	3.52	N/A	2.04	**± 5.0%**
% recovery of known impurities in six spiked samples	104.3	N/A	99.1	**90.0-110.0**
% RSD of known impurities in six spiked samples.	4.2	N/A	2.6	**NMT 10.0**

% RSD of known impurities in 12 replicate samples by Analyst-1 & Analyst-2	3.4	N/A	2.0	

LIMIT OF DETECTION AND LIMIT OF QUANTITATION:

Limit of Detection (LOD)

The limit of detection was determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte could be reliably detected with a signal to noise ratio of about 3:1. Resulted chromatogram was shown figure-24 and data was shown in the table no: 16.

LOD Solution:

Figure-24 Chromatogram for LOD

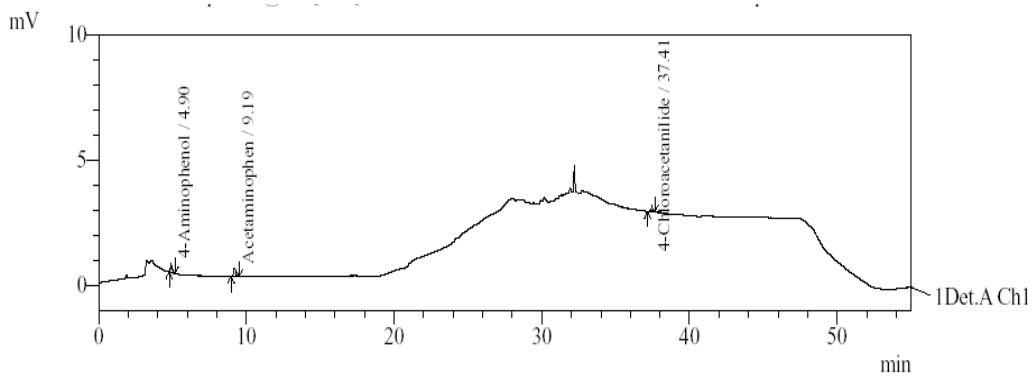


Table No: 16 Data for LOD

Name	Concentration in ppm	% RSD	Signal to noise ratio
4-aminophenol	0.04	2.5	3.8
4-Chloroacetanilide	0.01	2.5	4.1
Acetaminophen	0.02	1.0	3.3

Limit of Quantitation (LOQ):

The limit of quantitation was demonstrated by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte could be determined with acceptable accuracy and precision with a signal to noise ratio of 10:1. Resulted chromatogram was shown figure-25 and data was shown in the table no 17.

LOQ Solution:

Figure-25 Chromatogram for LOQ

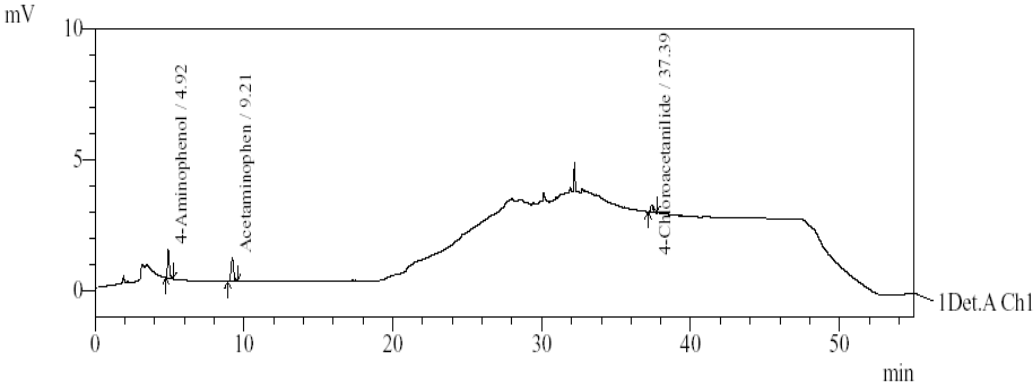


Table No: 17 Data for LOQ

Name	Concentration in ppm	% RSD	Signal to noise ratio
4-aminophenol	0.12	1.0	11.1
4-Chloroacetanilide	0.03	0.6	10.4
Acetaminophen	0.06	2.1	11.0

Acceptance criteria:

1) The signal to noise ratio should be 3:1 for LOD and 10:1 for LOQ.

2) The % Relative standard deviation for six replicate LOQ level areas should be NMT 10%.

LINEARITY AND RANGE

Linearity &Range

Standard solutions of Acetaminophen, 4-Aminophenol and 4-Chloroacetanilide in the concentration levels from 50 % to 150 % of standard solution were injected into HPLC system. The linearity graph was plotted from 50 % to 150% of drug concentration. Resulted chromatograms were shown figures-26,27,28,29,30,31,32,33 and data was shown in the tables no18,19,20,21,22,23.

Acceptance criteria

a. The correlation coefficient (r) must be NLT 0.999.
b. The RSD of replicate injections for lower and upper level concentrations should not be more than 2.0 %.

Figure -26 Chromatograms for linearity-50%

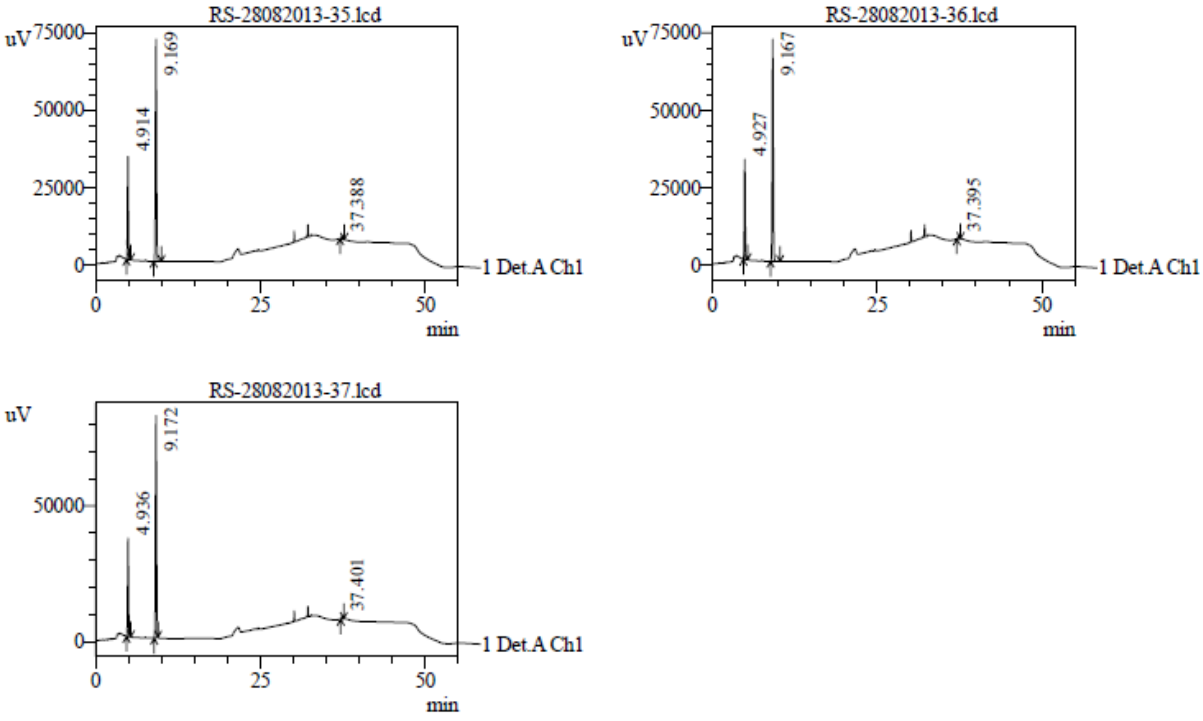


Figure -27 Chromatograms for linearity-75%

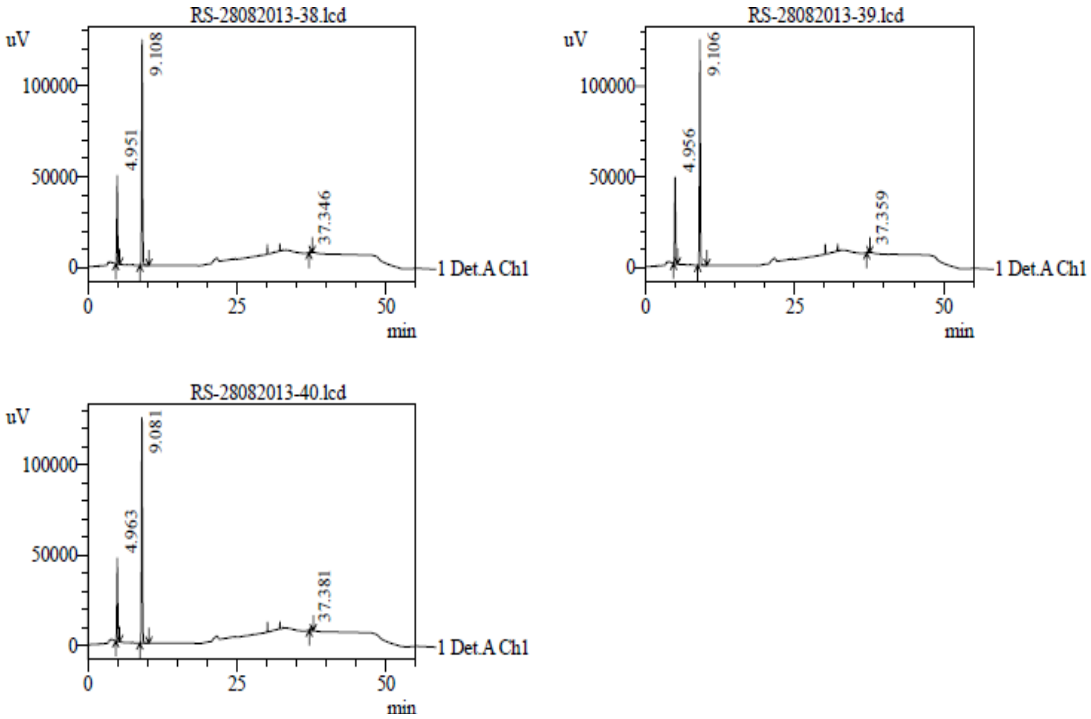


Figure-28 Chromatograms for linearity-100%

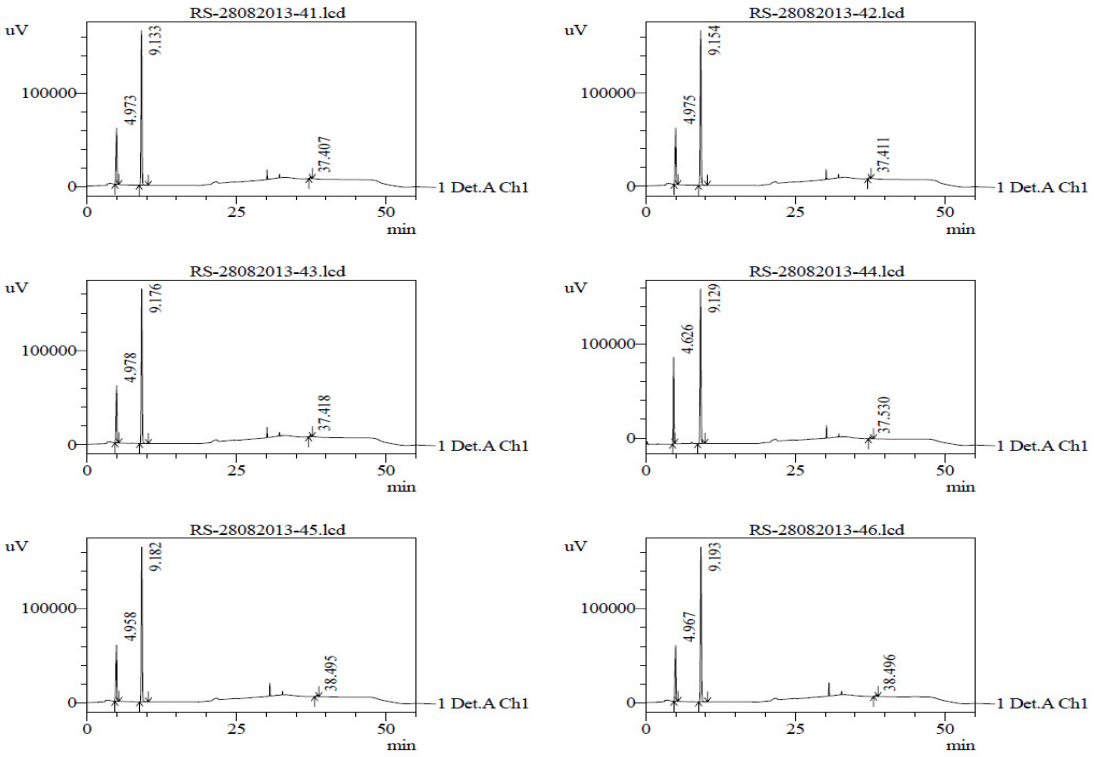


Figure -29 Chromatograms for linearity-125%

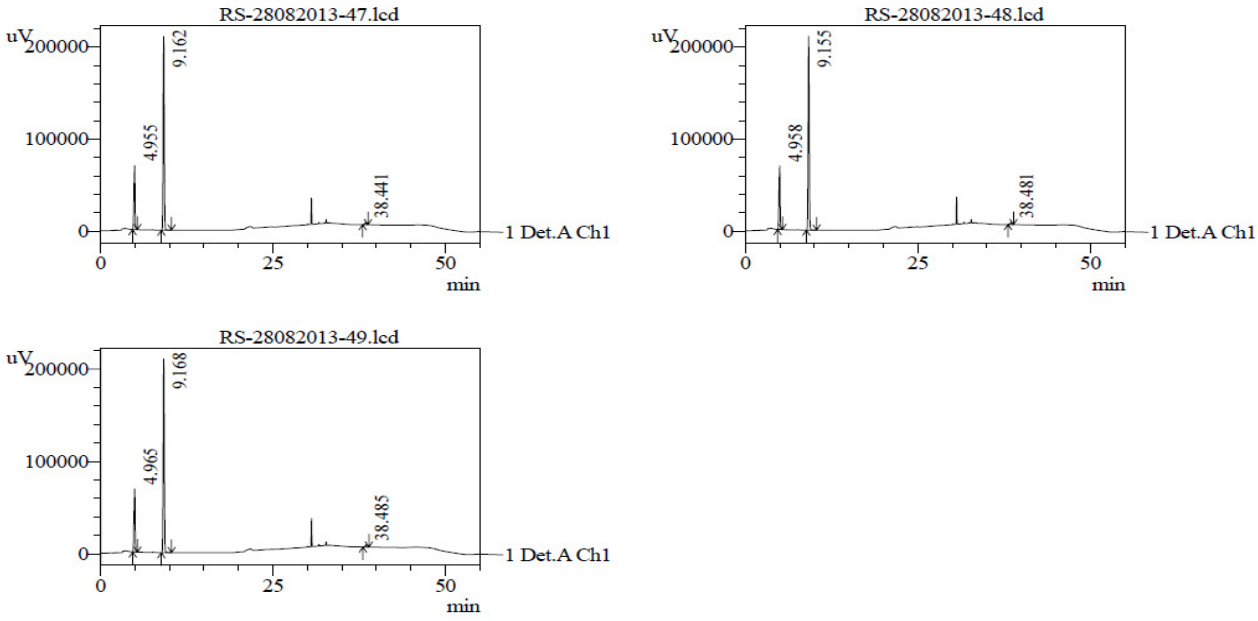


Figure -30 Chromatograms for linearity-150%

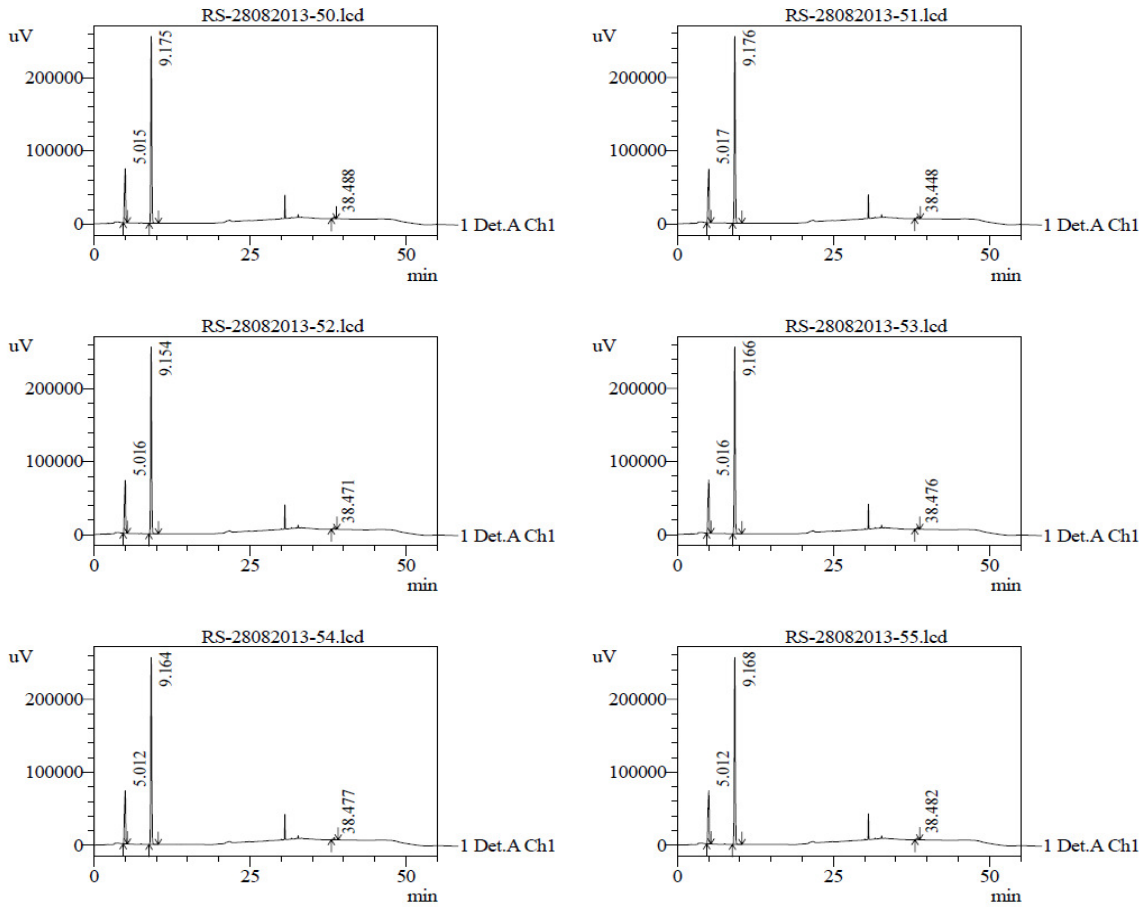


Figure- 31 Linearity curve for Acetaminophen

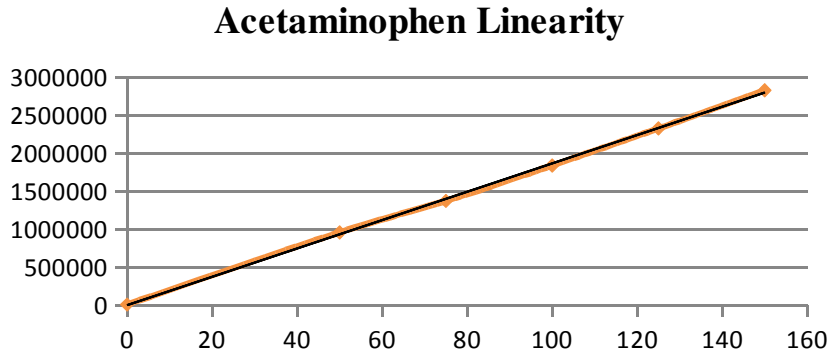


Table No: 18 Data for linearity (Acetaminophen)

Sample	Weight in mg	Peak Area	Conc. in %
Sample 2	50.76	5317	LOQ
Sample 3		954889	50
		1369691	75
Sample 4		1836781	100
Sample 5		2323373	125
Sample 6		2825739	150

Table No: 19 Results of linearity (Acetaminophen)

Acceptance criteria:

System Suitability Parameters	Results	Acceptance criteria
Correlation coeff. (r)	0.999	**NLT 0.999**
The % RSD of six replicate injection Lower Middle Higher	2.1 0.2 0.0	**NMT 2.0**
% of y-Intercept	-0.1	**±2.0**

Figure-32 Linearity curve for 4-Aminophenol

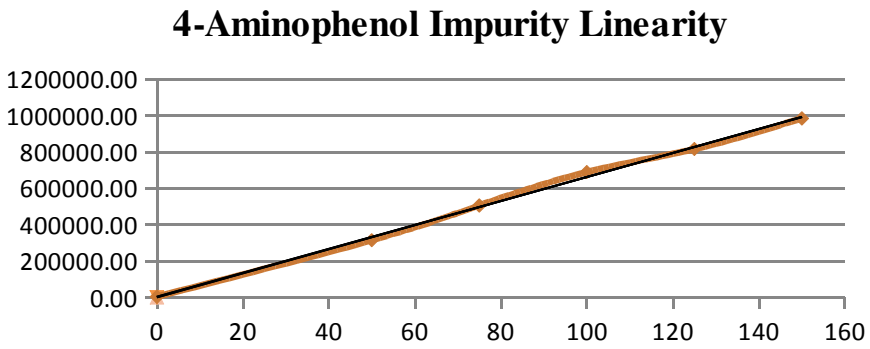


Table No: 20 Data for linearity (4-Aminophenol)

Sample	Weight in mg	Peak Area	Conc. in %
Sample 1	50.18	4329	LOQ
Sample 2		314922	50
Sample 3		506104	75
Sample 4		689002	100
Sample 5		816023	125
Sample 6		984526	150

Table No: 21 Results of linearity (4-Aminophenol)

System Suitability Parameters	Results	Acceptance criteria
Correlation coeff. (r)	0.999	**NLT 0.999**
The % RSD of six replicate injection Lower Middle Higher	 1.0 0.7 0.7	**NMT 2.0**
% of y-Intercept	0.5	**±2.0**

Figure- 33 Linearity curve for 4-Chloroacetanilide

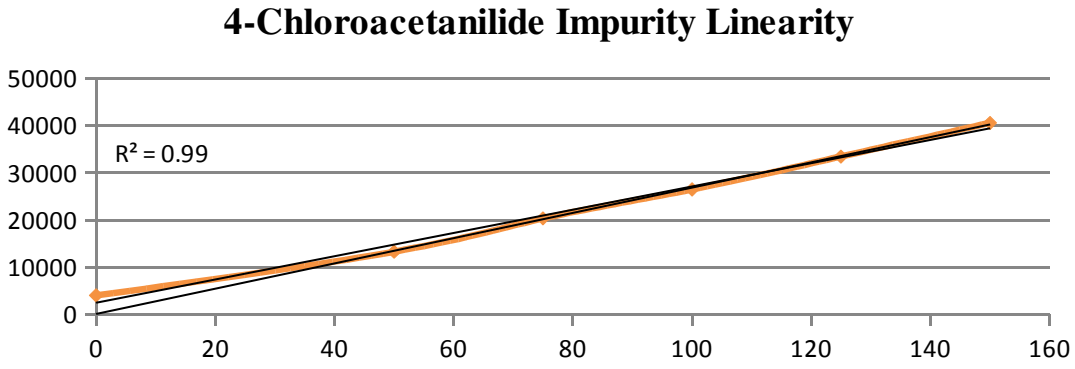


Table No: 22 Data for linearity (4-Chloroacetanilide)

Sample	Weight in mg	Peak Area	Conc. In %
Sample 1	20.34	3939	LOQ
Sample 2		13219	50
Sample 3		20265	75
Sample 4		26434	100
Sample 5		33390	125
Sample 6		40517	150

Table No: 23 Results of linearity (4-Chloroacetanilide)

System Suitability Parameters	Results	Acceptance criteria
Correlation coeff. (r)	0.999	**NLT 0.999**
The % RSD of six replicate injection Lower Middle Higher	0.6 1.9 1.7	**NMT 2.0**
% of y-Intercept	-1.2	**±2.0**

ACCURACY

Study of this analytical method was carried out by were drug concentration in the range of 50%-150%.resulted chromatograms were shown in the figures-34,35,36,37 and data was shown in the table no.24,25,26.

Figure -34 Chromatogram for sample of LOQ concentration

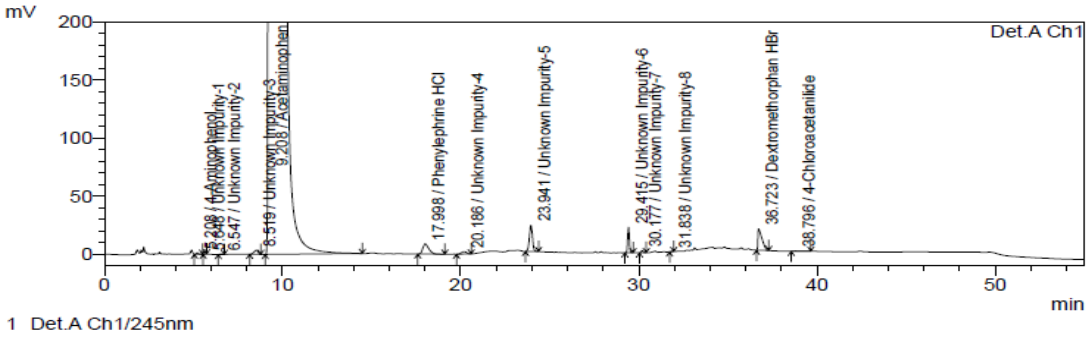


Figure-35 Chromatogram for sample of 50% concentration

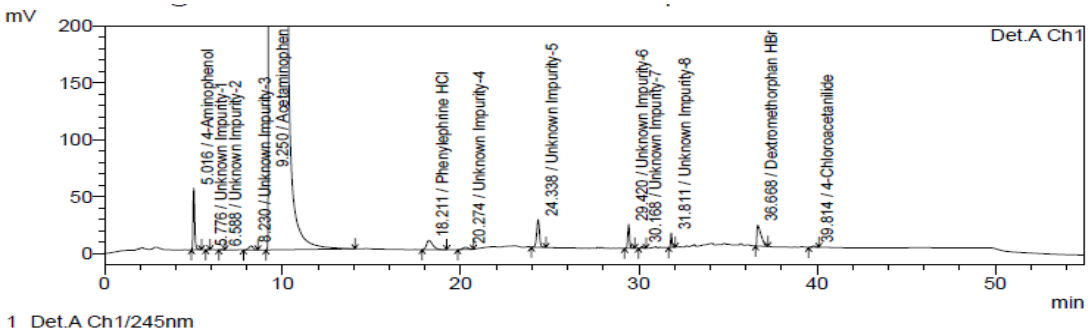


Figure-36 Chromatogram for sample of 100% concentration

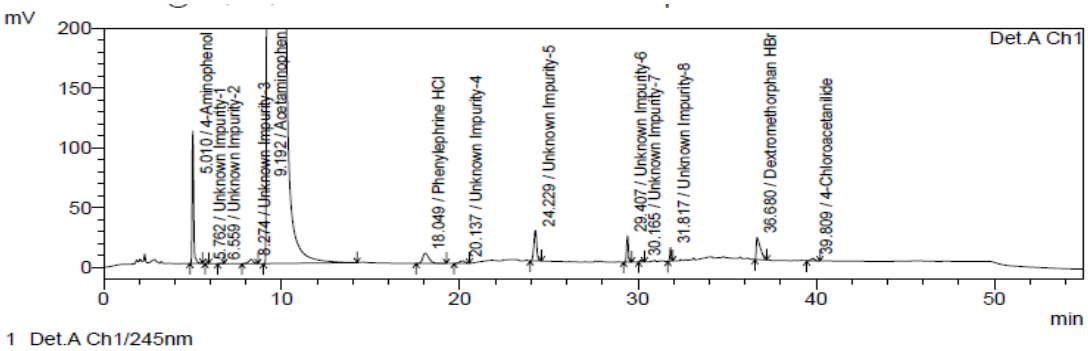


Figure-37 Chromatogram for sample of 150% concentration

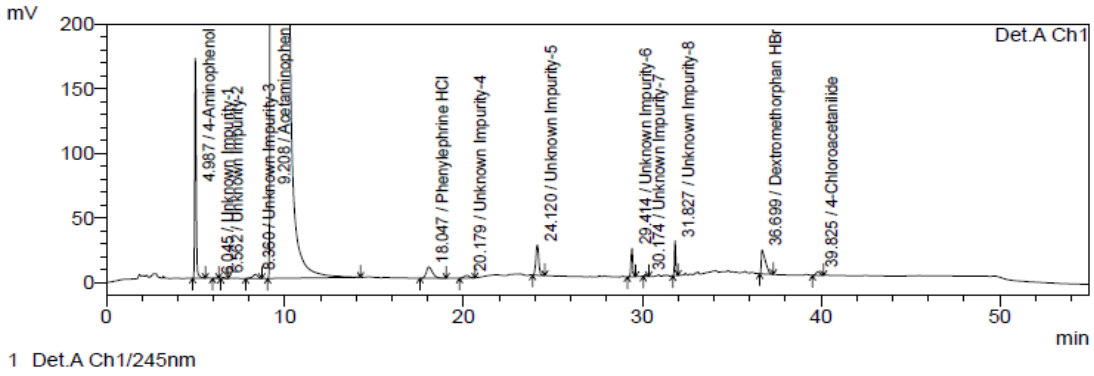


Table no: 24 Data for accuracy (4-aminophenol)

S. No	Accuracy Level	Sample Added in mg	Recovery in %	Average Recovery in %	RSD in %
01	LOQ Sol	8046.53	103.6	103.1	0.4
	LOQ Sol	8034.96	102.6		
	LOQ Sol	8017.15	103.0		
02	50.0%	8021.71	103.9	103.5	0.3
	50.0%	8030.25	103.1		
	50.0%	8027.96	103.6		
03	100.0%	8028.12	101.4	101.4	0.4
	100.0%	8052.44	100.9		
	100.0%	8040.55	102.0		
04	150.0%	8031.30	100.8	100.9	0.3
	150.0%	8013.89	101.2		
	150.0%	8020.06	100.6		
			Average	**102.2**	
			SD	**1.3**	
			% RSD	**1.3**	

Table no.25 Data for accuracy (4-Chloroacetanilide)

S. No	Accuracy Level	Sample Added in mg	Recovery in %	Average Recovery in %	RSD in %
01	LOQ Sol	8046.53	102.9	105.5	1.8
	LOQ Sol	8034.96	106.2		
	LOQ Sol	8017.15	107.3		
02	50.0%	8021.71	98.7	100.4	1.3
	50.0%	8030.25	100.4		
	50.0%	8027.96	102.0		
03	100.0%	8028.12	98.1	99.0	0.7
	100.0%	8052.44	99.8		
	100.0%	8040.55	99.2		
04	150.0%	8031.30	97.4	98.8	1.5
	150.0%	8013.89	98.1		
	150.0%	8020.06	100.9		
Average				**100.9**	
SD				**3.13**	
% RSD				**3.1**	

Table no.26 Results data for accuracy

System Suitability Parameters	Results			Acceptance criteria
	4-Aminophenol	Acetaminophen	4-Chloroacetanilide	
Tailing factor for Acetaminophen, 4-	1.0	1.0	1.0	NMT 2.0

Aminophenol and 4-Chloroacetanilide peaks from first standard injection.				
Theoretical plate count for Acetaminophen, 4-Aminophenol and 4-Chloroacetanilide peaks from first standard injection.	11374	16164	202495	**NLT 2000**
The %RSD of RT for six replicate injection of standard solution (for 4-Aminophenol, Acetaminophen and 4-Chloroacetanilide)	0.5	0.1	0.0	**NMT 1.0**
The %RSD of Peak response from six replicate injection of standard solution (for 4-Aminophenol, Acetaminophen and 4-Chloroacetanilide)	0.8	0.4	0.8	**NMT 2.0**
Resolution between 4-Aminophenol and Acetaminophen injection from first standard injection.	18.9		9.0	**NLT 2.0**
The % Recovery at LOQ level	103.1	N/A	105.5	**80.0 - 120.0**
The% Recovery at 50% to 150% level	103.5 101.4 100.9	N/A	100.4 99.0 98.8	**90.0-110.0**
% RSD of recovery	1.2	N/A	3.1	**NMT10.0**

ROBUSTNESS

Robustness was performed for this analytical method by chromatograms selected Parameters as given below and resulted chromatograms were shown in the figures-38 to 49 and data was shown in the table no:27to29.

Method Parameters:

1. Flow Rate

(Normal flow is 1.0 mL/min)

a. Reduced flow ® 0.8 mL/min

b. Increased flow ® 1.2 mL/min

2. Column Operating Temperature

(Normal temperature is 30 °C)

a. Reduced Temperature ® 28 °C

b. Elevated Temperature ® 32 °C

3. wavelength

(Normal wavelength is 245nm)

a. Reduced wavelength ® 243nm

b. Elevated wavelength ® 247nm

Acceptance Criteria

All the system suitability requirements must be met.

Figure- 38 chromatogram for standard with reduced Flow rate

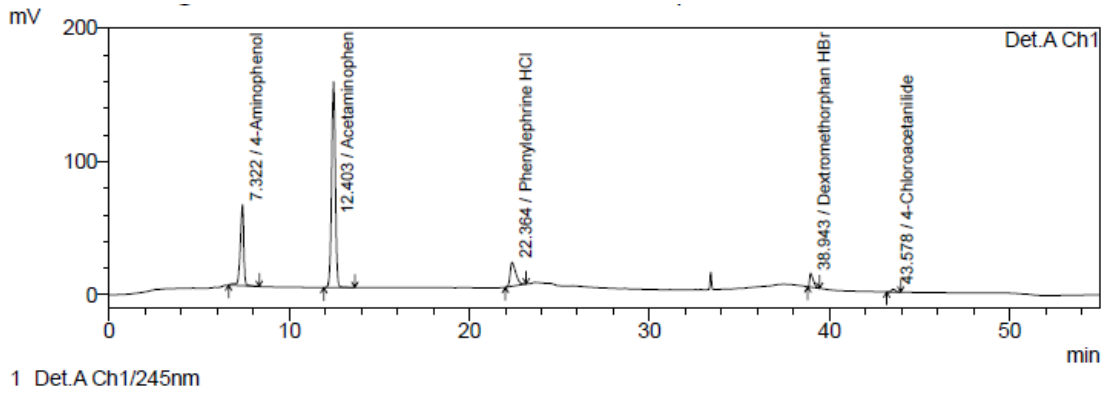


Figure -39 Chromatogram for Spiked sample with reduced Flow rate

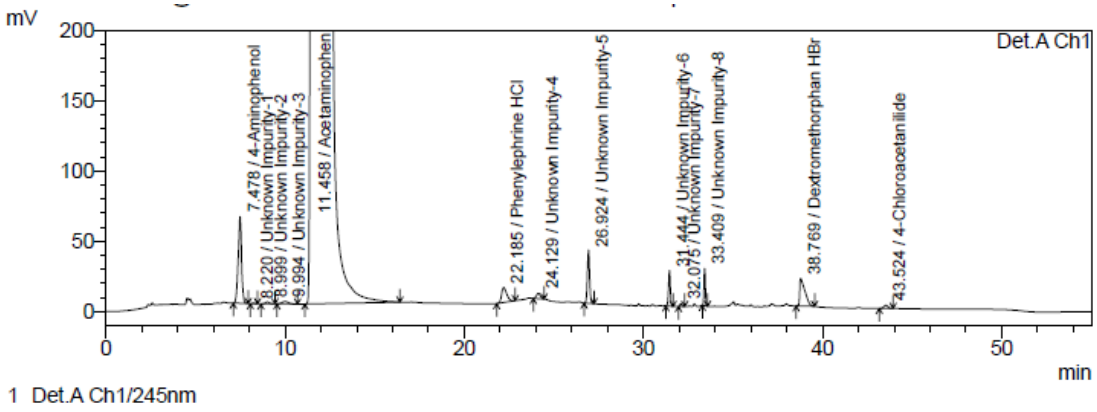


Figure-40 Chromatogram for standard with increased Flow rate

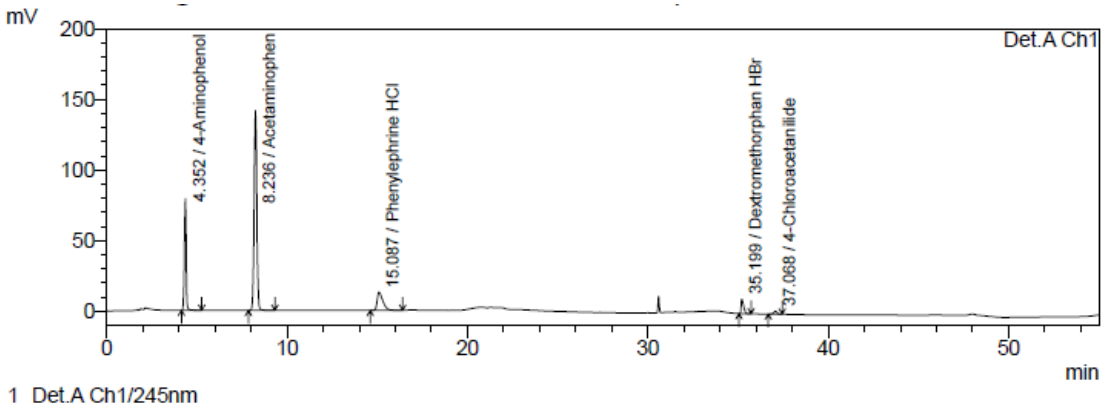


Figure -41 Chromatogram for sample with increased Flow rate

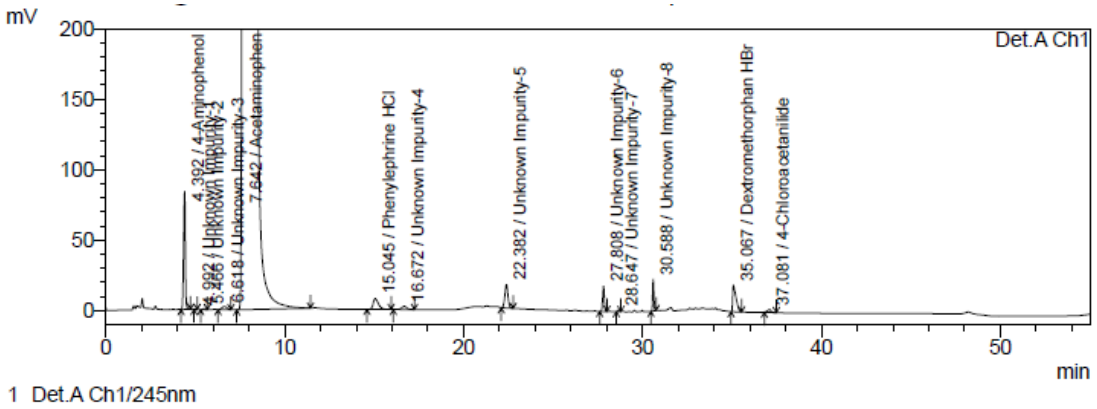


Table no. 27 Results for Flow rate parameter

System Suitability Parameters	Results			Acceptance criteria
	4-aminophenol	**Acetaminophen**	**4-Chloroacetanilide**	
Tailing factor for Acetaminophen, 4-Aminophenol and 4-Chloroacetanilide peaks from first standard injection. 1. 0.8 ml/min 2. 1.0 ml/min 3. 1.2 ml/min	 1.0 1.1 0.9	 1.0 1.0 0.9	 1.0 1.0 0.9	**NMT 2.0**
Theoretical plate count for Acetaminophen, 4-Aminophenol and 4-Chloroacetanilide peaks from first standard injection. 1. 0.8 ml/min 2. 1.0 ml/min 3. 1.2 ml/min	 8869 6865 8165	 17178 10374 13896	 170545 145149 212469	**NLT 2000**
The %RSD of RT for six replicate injection of standard solution (for 4-Aminophenol, Acetaminophen and 4-Chloroacetanilide) 1. 0.8 ml/min 2. 1.0 ml/min 3. 1.2 ml/min	 0.0 0.2 0.1	 0.2 0.3 0.1	 0.0 0.1 0.0	**NMT 1.0**

	The %RSD of Peak response from six replicate injection of standard solution (for 4-Aminophenol, Acetaminophen and 4-Chloroacetanilide) 1. 0.8 ml/min 2. 1.0 ml/min 3. 1.2 ml/min	 0.8 0.8 0.4	 1.3 0.2 0.6	 1.7 0.7 0.8	**NMT 2.0**
	Resolution between 4-Aminophenol and Acetaminophen injection from first standard injection. 0.8 ml/min 1.0 ml/min 1.2 ml/min	 18.8 14.7 16.5	N/A	N/A	**NLT 2.0**
	% recovery of known impurities 0.8 ml/min 1.0 ml/min 1.2 ml/min	 98.1 102.5 104.4	N/A	 99.6 101.2 102.8	**90.0-110.0**
	The % RSD between the results of known impurities obtained with three different flow rates.	3.2	N/A	0.9	**NMT 10.0**

Figure -42 Chromatogram for standard with reduced Temperature

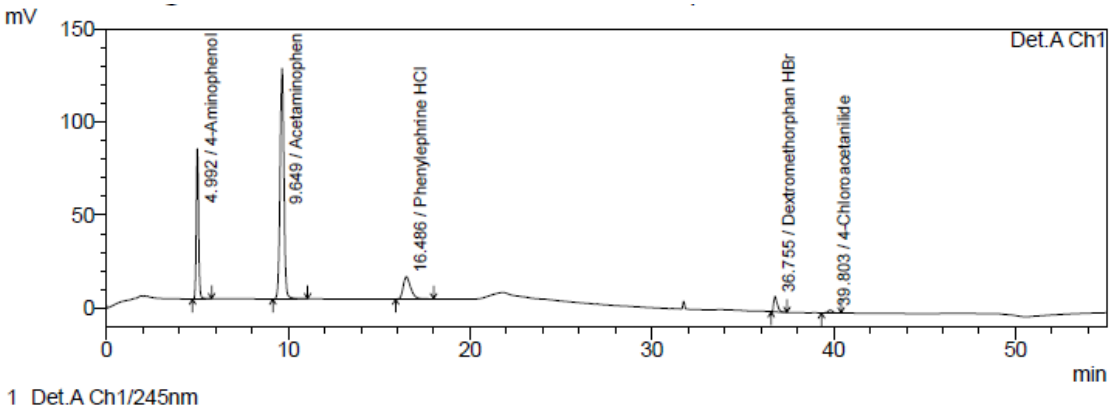


Figure -43 Chromatogram for sample with reduced Temperature

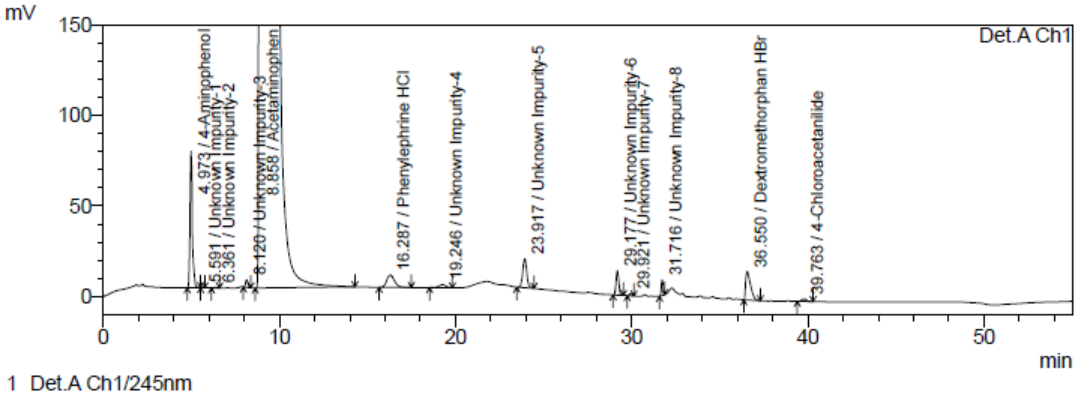


Figure-44 Chromatogram for standard with increased Temperature

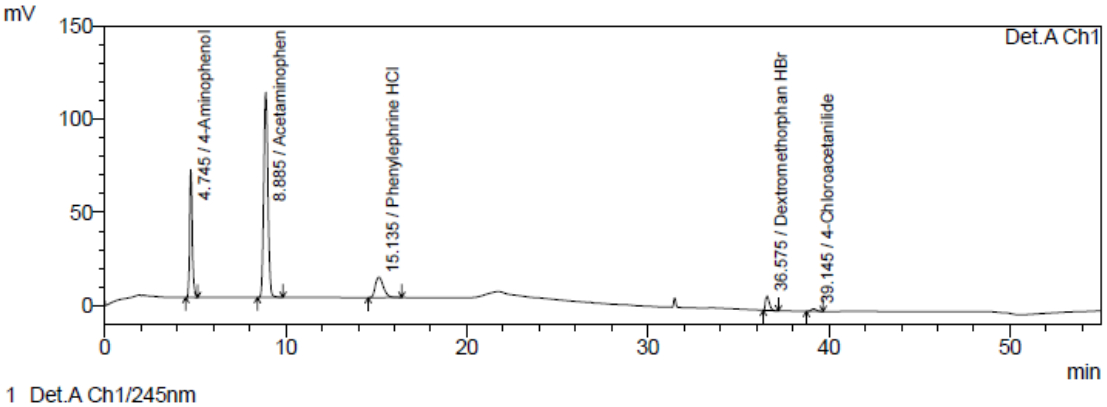


Figure -45 Chromatogram for sample with increased Temperature

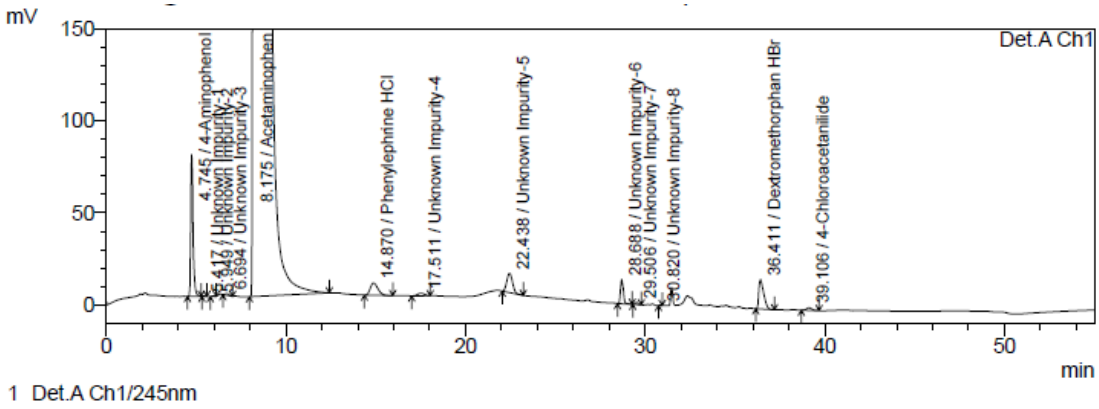


Table no.28 Results for Temperature parameter

System Suitability Parameters	Results			Acceptance criteria
	4-aminophenol	Acetaminophen	4-Chloroacetanilide	
Tailing factor for Acetaminophen, 4-Aminophenol and 4-Chloroacetanilide peaks from first standard injection. 1. 28°C 2. 30°C 3. 32°C	1.1 1.1 1.4	1.0 1.0 1.1	1.1 1.0 1.1	**NMT 2.0**
Theoretical plate count for Acetaminophen, 4-Aminophenol and 4-Chloroacetanilide peaks from first standard injection. 1. 28°C 2. 30°C 3. 32°C	6995 6865 4976	9711 10374 7011	125989 145149 97468	**NLT 2000**
The %RSD of RT for six replicate injection of standard solution (for 4-Aminophenol, Acetaminophen and 4-	0.1 0.2 0.0	0.1 0.3 0.0	0.0 0.1 0.0	**NMT 1.0**

	Chloroacetanilide)				
1.	28°C				
2.	30°C				
3.	32°C				
	The %RSD of Peak response from six replicate injection of standard solution (for 4-Aminophenol, Acetaminophen and 4-Chloroacetanilide)				
1.	28°C	0.5	1.7	0.9	**NMT 2.0**
2.	30°C	0.8	0.2	0.7	
3.	32°C	0.2	0.7	0.9	

	Resolution between 4-Aminophenol and Acetaminophen injection from first standard injection.				
1.	28°C	14.8	N/A	N/A	**NLT 2.0**
2.	30°C	14.7			
3.	32°C	11.9			
	% recovery of known impurities				
1.	28°C	99.2	N/A	100.1	**90.0-110.0**
2.	30°C	102.5		101.2	
3.	32°C	99.0		102.4	
	The % RSD between the results of known impurities obtained with three different temperatures.	2.0	N/A	1.1	**NMT 10.0**

Figure -46 Chromatogram for standard with reduced wavelength

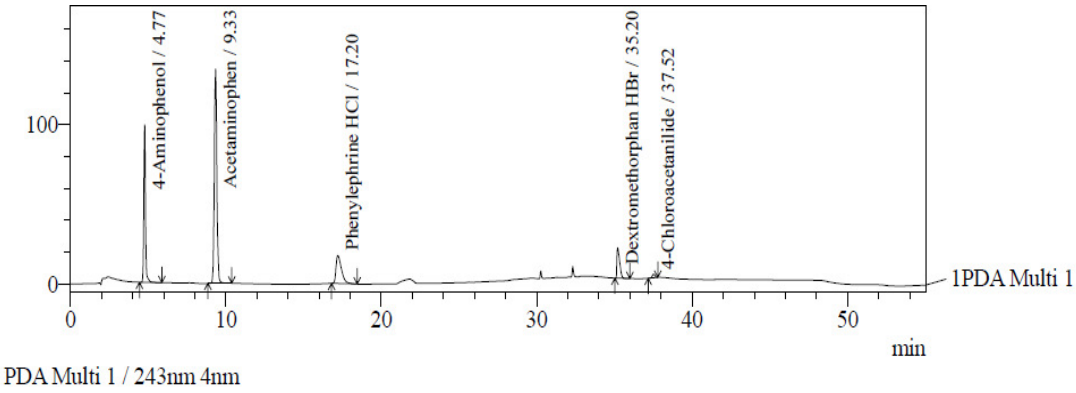


Figure -47 chromatogram for sample with reduced wavelength

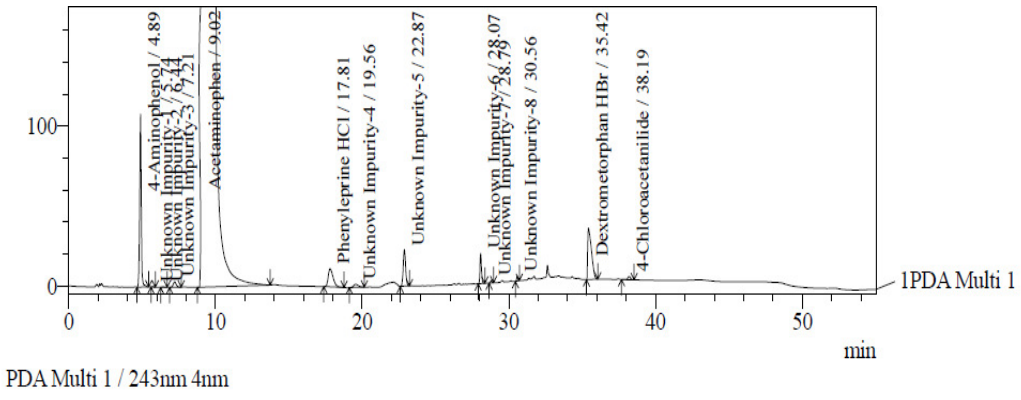


Figure-48 Chromatogram for standard with increased wavelength

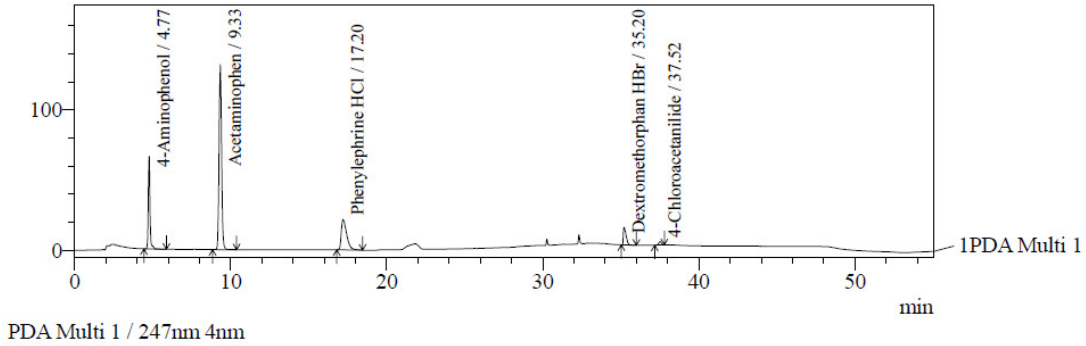


Figure-49 chromatogram for sample with increased wavelength

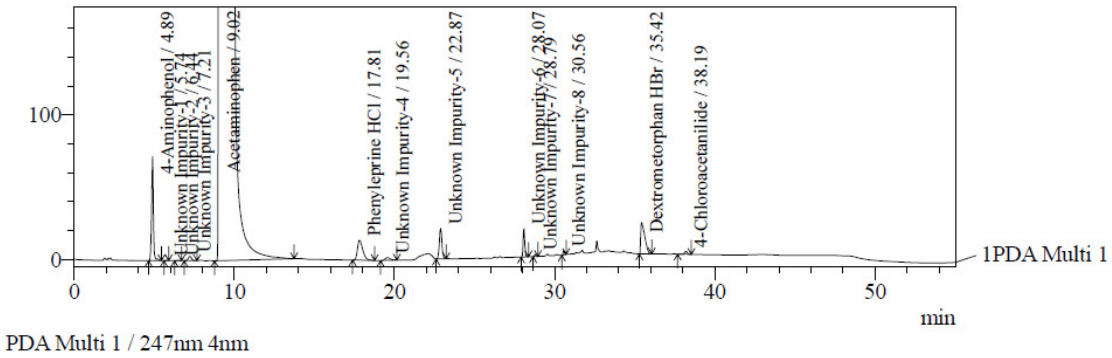


Table -29 Results for wavelength parameter

System Suitability Parameters	Results			Acceptance criteria
	4-aminophenol	**Acetaminophen**	**4-Chloroacetanilide**	
Tailing factor for Acetaminophen, 4-Aminophenol and 4-Chloroacetanilide peaks from first standard injection. 1. 243nm 2. 245nm 3. 247nm	 1.0 1.0 1.2	 1.1 1.1 1.1	 1.1 1.1 1.1	**NMT 2.0**
Theoretical plate count for Acetaminophen, 4-Aminophenol and 4-Chloroacetanilide peaks from first standard injection. 1. 243nm 2. 245nm	 8554 9550 8482	 14595 15655 14593	 211765 219780 213126	**NLT 2000**

3. 247nm				
The %RSD of RT for six replicate injection of standard solution (for 4-Aminophenol, Acetaminophen and 4-Chloroacetanilide) 1. 243nm 2. 245nm 3. 247nm	 0.0 0.0 0.0	 0.1 0.2 0.1	 0.0 0.0 0.0	 **NMT 1.0**

The %RSD of Peak response from six replicate injection of standard solution (for 4-Aminophenol, Acetaminophen and 4-Chloroacetanilide) 1. 243nm 2. 245nm 3. 247nm	 0.5 0.5 0.5	 0.1 0.1 0.1	 0.6 1.3 1.1	 **NMT 2.0**
Resolution between 4-Aminophenol and Acetaminophen injection from first standard injection. 1. 243nm 2. 245nm 3. 247nm	 17.7 18.7 17.7	 N/A	 N/A	 **NLT 2.0**
% recovery of known impurities 1. 243nm	 101.2	 N/A	 103.1	 **90.0-110.0**

2. 245nm 3. 247nm	100.7 99.1		96.7 100.2	
The % RSD between the results of known impurities obtained with three different Wavelengths.	1.1	N/A	3.5	**NMT 10.0**

8. RESULTS AND DISCUSSION

The method was developed with gradient program of mobile phase A was 1-pentane sulphonic acid sodium salt buffer pH 6.0 (Buffer-1) and methanol (85:15) v/v and mobile phase B was potassium dihydrogen orthophosphate buffer pH 3.0 (Buffer-2) and acetonitrile in the ratio (70:30) v/v with flow rate 1.0 ml/min on Zodiac C-18, (250 × 4.6 mm, 5 µ) with UV detection at 245 nm gave a satisfactory chromatogram with run time 55 min.

The present proposed system provides shorter analysis time and conserves mobile phase system. The method was validated based on United States pharmacopoeia and ICH parameters. The parameters are accuracy, precision, LOD, LOQ linearity, range specificity, ruggedness and robustness.

Results of validation parameters for 4-Aminophenol:

s.no	Parameter	Experiment	Result	Specification
1.	**System suitability**	**%RSD**	0.4	The % RSD of standard area should be NMT 2.0
2	Specificity	Placebo interference	Nill 0.4	The Placebo should not show any peak at the retention time of 4-Aminophenol, Acetaminophen and 4-Chloroacetanilide peaks.
3.	Precision	System Precision	0.4	The % RSD of standard should be NMT 2.0
		Method Precision	0.8 0.42	The % RSD of Impurity value obtained in spiked samples should not be more than 10.0. The Confidence limits should be ± 5.0%
		Intermediate Precision	4.2 3.52	The % RSD of Impurity value obtained in spiked samples not more than 10.0. The Confidence limits should be ± 5.0%.
4.	Ruggedness	System to System Variability	2.1	The % RSD of Impurity value obtained in spiked samples should not be more than 10.0.
		Column to Column Variability	2.1	The % RSD of Impurity value obtained in spiked samples should not be more than 10.0.
		Analyst to Analyst qualification	2.1	The % RSD of Impurity value obtained in spiked samples should not be more than 10.0.
5.	Lod	Detection of limit	2.5	The %RSD should not more than 20.0
6.	Loq	Quantification of limit	1.0	The % RSD should not more than 10.0
7.	Linearity and Range	Coefficient of correlation (r)	0.999 0.5	The correlation co-efficient (r) should not be less than 0.999. % of y-Intercept should be ±2.0
8.	Accuracy	Recovery	103.1 102.2 1.3	The % Recovery at LOQ level 80.0% - 120.0%.The % Recovery at 50% to 150% level 90.0% - 110.0%. RSD should not be more than 10.0%.
9.	Robustness	Effect of variation temperature	2.0	The % RSD of Impurity value obtained in spiked samples should not be more than 10.0.
		Effect of variation in flow rate	3.2	The % RSD of Impurity value obtained in spiked samples should not be more than 10.0.
		Effect of variation in wavelength	1.1	The % RSD of Impurity value Obtained in spiked samples should not be more than 10.0 .

Results of validation parameters for 4-Chloroacetanilide:

s.no	Parameter	Experiment	Results	Specification
1.	**System suitability**	**%RSD**	0.9	The % RSD of standard area should be NMT 2.0
2	Specificity	Placebo interference	Nill 0.4	The Placebo should not show any peak at the retention time of 4-Aminophenol, Acetaminophen and 4-Chloroacetanilide peaks.
3.	Precision	System Precision	0.9	The % RSD of standard should be NMT 2.0
		Method Precision	2.1 0.56	The % RSD of Impurity value obtained in spiked samples should not be more than 10.0. The Confidence limits should be ± 5.0%
		Intermediate Precision	2.6 2.04	The % RSD of Impurity value obtained in spiked samples not more than 10.0. The Confidence limits should be ± 5.0%.
4.	Ruggedness	System to System Variability	2.6	The % RSD of Impurity value obtained in spiked samples should not be more than 10.0.
		Column to Column Variability	2.6	The % RSD of Impurity value obtained in spiked samples should not be more than 10.0.
		Analyst to Analyst qualification	2.6	The % RSD of Impurity value obtained in spiked samples should not be more than 10.0.
5.	Lod	Detection of limit	2.5	The %RSD should not more than 20.0
6.	Loq	Quantification of limit	0.6	The % RSD should not more than 10.0
7.	Linearity and Range	Coefficient of correlation (r)	0.999 -1.2	The correlation co-efficient (r) should not be less than 0.999. % of y-Intercept should be ±2.0
8.	Accuracy	Recovery	105.5 100.9 3.1	The % Recovery at LOQ level 80.0% - 120.0%. The % Recovery at 50% to 150% level 90.0% - 110.0%. RSD should not be more than 10.0%.
		Effect of		The % RSD of Impurity value obtained in

9.	Robustness	variation temperature	2.0	spiked samples should not be more than 10.0.
		Effect of variation in flow rate	0.9	The % RSD of Impurity value obtained in spiked samples should not be more than 10.0.
		Effect of variation in wavelength	1.1	The % RSD of Impurity value Obtained in spiked samples should not be more than 10.0 .

9. CONCLUSION

As per the aim of present work, the RP-HPLC method was developed to estimate the related substances of acetaminophen in acetaminophen, phenylephrine Hcl and dextromethorphan HBr in soft gelatin capsules. From the result of validation parameters obtained, it was observed that the developed method was proven to be specific, precise, linear, accurate, rugged and robust and is suitable for its intended purpose.

Hence it was concluded that this method could be used for the routine estimation of related substances of acetaminophen in acetaminophen, phenylephrine Hcl, dextromethorphan HBr in soft gelatin capsules.

BIBLIOGRAPHY

1. D. Kealey and P. J. Haines. Analytical Chemistry. 1st edition. New Delhi: Viva Brook Pvt Ltd. 2002; P.1-3.
2. Skoog, West holler crouch. Fundamentals of Analytical Chemistry. 8th edition. Australia: Thomason Brooks/Cole. 2004; P. 2-4.
3. Willard, Merit, Dean, Settle. Instrumental Method of Analysis. 7thedition. New Delhi: CS publishers and distributors. 1986; P. 1, 592,622-628, 256-264.
4. Sharma BK. Organic Spectroscopy. 6th edition. New Delhi: Goel Publishing House. 2002; P.75.
5. Gurdeep Chatwal, Sham Anand. Instrumental Methods of Chemical Analysis. 5thedition. New Delhi: Himalaya Publishing House. 2002; P .25-67.
6. Sharma B.K. Instrumental Method of Chemical Analysis. 18th edition Meerut: Goel Publishing House. 1999; P. 1-23, 175-203.
7. Jeffery G. H, Denny R. C, Barnes J. D, Thomas M. Vogel's text book of Quantitative Chemical Analysis. 6thedition. Pearson Education. P. 2-7, 216-227.
8. Munson JW. Pharmaceutical Analysis Modern Methods. Part B. Bombay: International Medical book distributors. 2001; P . 15-54, 62-63.
9. Snyder L.R, Kirkiland J. J. Practical HPLC method development. Newyork: Wiley interscience publication. 1997; P. 87-135, 210.
10. Hdico stas E, wendawiak B.W. Koch M. Quality Assurance in Analytical Chemistry Training and Teaching. 1stedition. New Delhi: Springer Pvt Ltd. International Springer Group. 2004; P. 204-219.
11. Martindale. The Complete Drug Reference. 3thedition. London: pharmaceutical press. 1999; P.128-523, 1027.
12. ICH-Q2A. Validation of Analytical Procedure / Methodology. Geneva, Switzerland: ICH Harmonised Triplicate Guidelines; 1996. P. 1-8.
13. Code ICH-Q2B. Validation of Analytical Procedure / Methodology. Geneva, Switzerland: ICH Harmonized Triplicate Guidelines; 1996. P. 1-8.
14. Quality Assurance guide. 1stedition. Organization of pharmaceutical producers of India. 2001; Section 7.1. P. 1-20.
15. United States of Pharmacopeia. 26th edition. United state Pharmacopoeial Convection. Rockville. 2003; P. 1151-1341, 1527.
16. In British Pharmacopeia. 2nd edition. Published by the Stationary Office on Behalf of the Medicines and Healthcare Products Regulatory Agency (MHRA) . 2011; P.1647, 1704-1705.

17. In British pharmacopeia. 1st edition. Published by the Stationary Office on Behalf of the Medicines and Healthcare Products Regulatory Agency (MHRA). 2011; P.665-667.

18. The Merck Index. An Encyclopedia of Chemicals, Drugs and Biologoical. 9th edition. Rahway. N. J., U.S.A: Merck&co., INC; 2006.P.472, 2083.

19. Shepherd DB, Barker WM, Hargrave JJ, MarkhamAptalis MA. Development and Validation of an Acetaminophen Impurity Method. Pharmatech Inc. 2013; P.1.

20. Octavian Ca linescu, Irinel A, Badea1, Luminit , Vla descu, Viorica Meltzer and Elena Pincu. HPLC Separation of Acetaminophen and its Impurities Using A Mixed-modeReversed-Phase/Cation Exchange Stationary Phase. ChromSci . 2012; Vol 50: P. 335–342.

21. Eglal A, Abdelaleem, Nada S, Abdelwahab. Validated stability indicating RP-HPLC method for determination of paracetamol, methocarbamol and their related substances. Analytical Chemistry. 2012; Vol 5(2): P. 541-545.

22. Gnana Raja M, Geetha G, Sangaranarayanan A. Simultaneous Stability Indicating Method Development and Validation for Related Compounds of Ibuprofen and Paracetamol Tablets by RP-HPLC Method. J Chromat Separation Techniq. 2012; P. 3-8

23. PhaznaTA, Setti, Aravind, Srikanth S, Nallapeta, Sivaramaiah, Pawar, Smita C, Rao, Venkateshwara J. Method development and validation of paracetamol drug by RP-HPLC. J Med Allied Sci . 2013; Vol. 3 (1): P. 8-14.

24. Sadana Gangishetty, Surajpal Verma. RP-HPLC Method Development and Validation for Simultaneous Estimation of Clarithromycin and Paracetamol. ISRN Analytical Chemistry. 2013; Article ID 948547: P. 1-5.

25. Arunadevi S, Birajdar S N, Meyyanathan B, Suresh. Methode Development and validation for the simultaneous determination of paracetamol and tramadol in solid dosage by PR-HPLC. IJPRD. 2009; Vol-1(10): P. 1-5.

26. Palled Mahesh, Karagane Swapnaleel, Mane Aruna, Bhat Anilchandra, Shinde Prashanti Analytical Method Development And Validation Of Acetaminophen, Caffeine ,Phenylephrine Hydrochloride And Dextromethorphan Hydrobromide In Tablet Dosage Form By RP-HPLC. ISSN. 2013; Vol 2(2): P. 9-15.

27. Fuad Al-Rimawi. Normal-phase LC method for simultaneous analysis of pseudophedrine hydrochloride, dextromethorphan hydrobromide, chlorpheniramine maleate, and paracetamol in tablet formulations. SPJ. 2010; Volume 18(2): P 103–106.

28. İ. M. Palabıyık, F. Onur . The Simultaneous Determination of Phenylephrine Hydrochloride, Paracetamol, Chlorpheniramine Maleate and Dextromethorphan Hydrobromide in Pharmaceutical Preparations. Chromatographia. 2007; Vol 66 (1): P. 93-96.

29. Thummala V, Raghava Raju, Noru Anil Kumar, Seshadri Raja Kumar, Annarapu Malleswara Reddy et al., Development and Validation of a Stability-Indicating RP-HPLC Method for the Simultaneous Estimation of Guaifenesin and Dextromethorphan Impurities in Pharmaceutical Formulations. Chromatographic Research International. 2013; Article ID 315145: P.1-12.

30. Marin AE, Garcia A, Garcia C, Barbas. Validation of a HPLC quantification of acetaminophen, phenylephrine and chlorpheniramine in pharmaceutical formulations capsules and sachets. JPBA. 2002; Volume 29(4): P. 701–7140.

31. Rouhollah Heydari A. New HPLC Method for the Simultaneous Determination of acetaminophen, Phenylephrine, Dextromethorphan and Chlorpheniramine in Pharmaceutical Formulations. Analytical Letters. 2008; Vol 41(6): P. 965-976.

32. Marin AC. Barbas CE, versus. HPLC for the dissolution test in a pharmaceutical formulation containing acetaminophen, phenylephrine and chlorpheniramin. JPBA. 2004; Vol 35(4): P.769–777.

33. Shalini Joshi, C Bhatia, CS Bal. Quantization of Dextromethorphan and Levocetirizine in Combined Dosage form Using a Novel Validated RP-HPLC Method. Indian J Pharm Sci. 2012 ; Vol 74(1): P. 83–86.

34. Brahmbhatt, Kunal D, Bapna M, Shah SR, Patel, RA, Patel CM. Method Devlopment And Validation Of Paracetamol And Tramadol Hcl By RP-HPLC in Bulk And Pharmaceutical Dosage form. ISSN. 2013; Vol. 4(3): P. 79.

35. www.abcchemistry.com.

36. www.sciencedirect.com.